Docket No.: 0147-0262PUS1
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:
Klaus K NIELSEN et al.

Application No.: 10/507,355 Confirmation No.: 5659

Filed: June 9, 2005 Art Unit: 1638

For: METHOD OF REPRESSING FLOWERING IN A PLANT Examiner: Baum, Stuart F.

DECLARATION UNDER 37 C.F.R. § 1.131

MS AF
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

1. I, Dr. Christian Sig Jensen declare as follows:

2. I am a co-inventor of the subject matter disclosed and claimed in the above-identified U.S. Patent application (the "present application") and I am familiar with the prosecution history of the application.

3. I understand that the Examiner has made a rejection of the claims over Jensen et al. It is my understanding that Jensen et al. is effective under U.S. law as a prior art reference as of March 12, 2001.

4. The Jensen et al. reference published less than one year before the filing date of U.S. Provisional Application 60/363,125, filed March 11, 2002, to which this application claims priority.

5. The invention claimed in the present application was reduced to practice prior to March 12, 2001. As evidence of prior invention, the following are attached:

Exhibit 1 is a laboratory notebook page showing the first identification of the mRNA sequence of LpTFL1 comprising nucleotide 54 to nucleotide 929 of SEQ ID

NOs: 1 in the present application and codes for the amino acid sequence of SEQ ID NO: 3 in the present application.

Exhibit 2 is laboratory notebook pages showing the construction of the vectors E47-LpTFL1 for transformation into grasses and pCAMBIA3300-LpTFL1 for transformation into *Arabidopsis*.

Exhibit 3 is a diagram illustrating the construction of vector E47 based on the plasmid pAHC27 (Christensen and Quail, 1996).

Exhibit 4 is a copy of Christensen and Quail (1996) describing the pACH27 vector for monocot transformation

Exhibit 5 is a diagram illustrating the map of vector pCAMBIA3300

Exhibit 6 is a description of the CAMBIA binary vector system used for *Agrobacterium*-mediated transformation inclusive pCAMBIA3300.

Exhibit 7 is a lab notebook page describing the preparation of a plasmid for transformation into *Arabidopsis* (continued on page 16- see bottom of page 9). This page visually compares a wild-type *Arabidopsis* to a LpTFL1 transformant. The wild-type *Arabidopsis* shows flowering and a long stem, while the transformant is not flowering. The results confirmed that transformation and expression of LpTFL1 in a plant reduced or prevented flowering in the transformed plant.

Exhibit 8 shows the continuation of the transformation experiments of *Arabidopsis* wildtype and tfl1 mutant with LpTFL1 (as described in Jensen et al.).

Exhibit 9 shows the subsequent DNA gel blot analysis of transformed Arabidopsis plants.

Exhibit 10 shows the PCR verification of *Lolium perenne* callus lines transformed with LpTFL1.

Exhibit 11 is a print out of the excel log-file used to score the phenotypes of the LpTFL1 transformants in *Arabidopsis* after 200 days of growth. In preparation of the figures for the paper Jensen et al. 2001, plants that did not flower at this stage were set to a flowering time of 200 days although they remained non-flowering after the manuscript were submitted. Shown are also document properties including information on when the document was created and modified.

Exhibit 12 is a pre-publication dated draft of the Jensen et al. paper as sent to Plant Physiology by the present inventors prior to March 12, 2001. The experiments reported therein further evidence that the present inventors had reduced to practice a method for reducing flowering in a plant as presently claimed prior to the March 12, 2001 publication date of the Jensen et al. reference.

These experiments resulted in the preparation of a transgenic plant having reduced flowering as compared to the wild type plant. These experiments were conducted by transforming an *Arabidopsis* plant and a *Lolium perenne* with a nucleotide sequence encoding an amino acid sequence as shown in Figure 4 and SEQ ID NO:3 of the present application.

I hereby declare that all statements made herein of my own knowledge are believed to be true, and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Signed

16.01.09
Date

LRS/MHE/cjd

Exhibit 1

INTRODUCTION

In order to optimise the cDNA-adapter reaction described page 82 a combination of two enzymes in the same reaction was tried; the Superscript and the rTth.

PROCEDURE

The contents of the reactions and the procedure is outlined below:

	A	B
RNA (0.5 µg)	1.0 µl	1.0 µl
START primer	1.0 µl	2.0 µl
CAP	1.0 µl	2.0 µl
H_2O	2.0 µl	-

70°C 2 min → ice then add:

5x Superscript buff	2.0
DTT (20 mM)	1.0
dNTP	2.0
Superscript	1.0

↳ 42°C for 45 min then add:

KCl (500 mM)	0.8 µl
rTth enzyme	2.0 µl

→ 72°C 2 min → 37°C 15 min → 72°C 15 min

As control one Superscript reaction was made as on page 80 and one rTth reaction was made as on page 81. Reactions were then diluted with 40 µl TE buffer. 5 µl was then used as template in PCR amplification with the optimum conditions described page 82

RESULTS

Comparing the four RT reactions set up, it is clear, that the combination of using Superscript and rTth gives the best results.

However compared to the previous results the results are up to the same level.

The raising start primer concentrations does not seem to increase efficiency.

Continued on page 85

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Mark A. Brell — 29.01.99 — Signed — Date — Signed — Date

14

PROJECT Construction of Antisense and Sense LPTFL

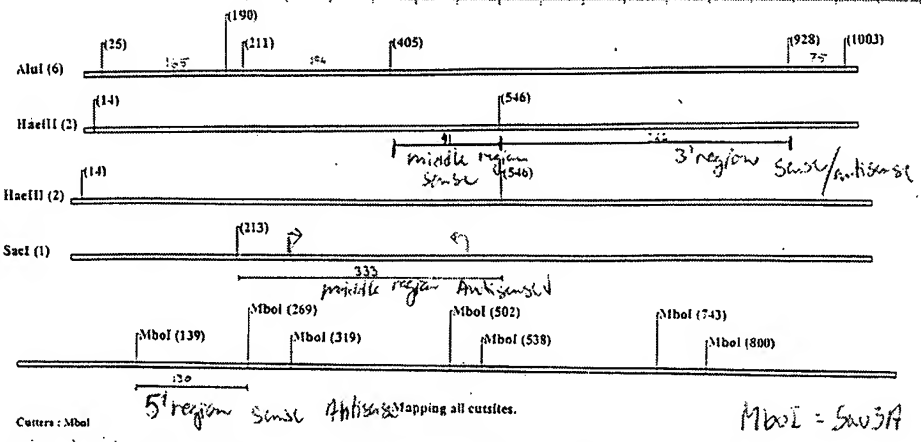
Notebook No. 377-B

Continued From Page

INTRODUCTION

The gene LpTFL (see page 10) is used for different antisense and sense constructions. The vector used is the pMST-AMC27-3 (E47) containing the Ubiquitin promotor with the Ex intron, the GUS and the NOS terminator.

First step is to remove the GUS with SmaI and SstI



For a full length sense and antisense LpTFL, a fragment was generated with PFU and two primers yielding a 880 bp fragment (blunt)

MboI = Sau3A

PROCEDURE

In order to generate fragments LpTFL was cut:

LpTFL (365 ng/µl)	12	12	12
AluI	2	–	–
HaeIII	2	2	–
SstI	–	2	–
Sau3A	–	–	3
BuffG	2	2	2
H_2O	2	2	3
	20.0	20.0	20.0

Vector was cut with:

E47 (15 µg)	30.0
SstI	3.0
SmaI	3.0
BuffH	6.0
H_2O	18.0
incubate	37°C for 2 hr.

Samples were incubated at 37°C for 2 hr.

Continued on Page 15

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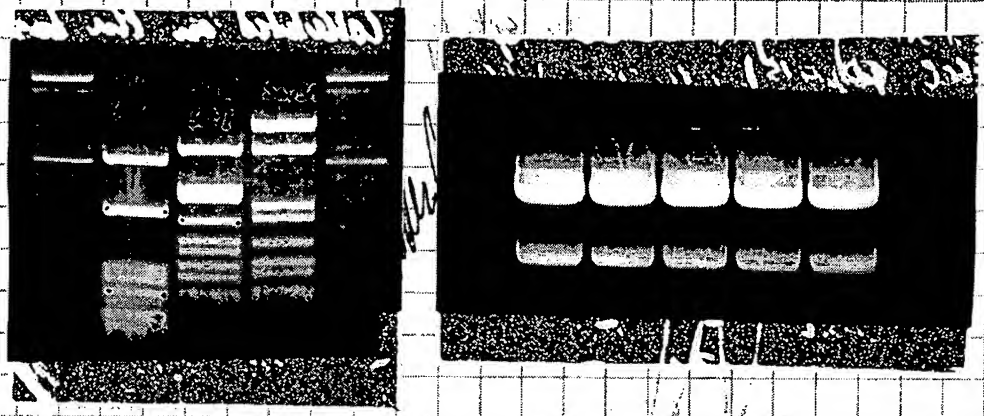
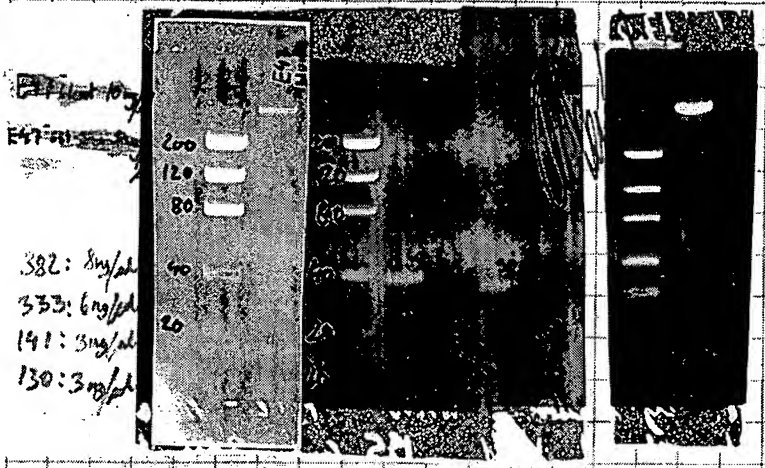
All the fragments were run on a 1.5% agarose TBE gel and the bands excised



Fragments and plasmid were purified and eluted from Qiagen spin columns

Small fragments were loaded alongside a DNA mass ladder to predict conc.



On the basis of these results the molar conc. was calculated:

LPTFL 880	(2.7 fmol/ng × 70 ng/μl)	=	182.4 fmol/μl
382	(4.0 fmol/ng × 8 ng/μl)	=	32.0 fmol/μl
333	(4.6 fmol/ng × 6 ng/μl)	=	27.6 fmol/μl
141	(10.3 fmol/ng × 3 ng/μl)	=	32.7 fmol/μl
130	(11.8 fmol/ng × 3 ng/μl)	=	35.4 fmol/μl
E47⁻ (Bst ip)	(0.44 fmol/ng × 80 ng/μl)	=	35.0 fmol/μl

— NB after blunting with Pfu.

In order to remove 3'-overhang from the SstI site on E47⁻ a Pfu polishing reaction was performed:

E47⁻	46.0
Buffer	6.0
dNTP	6.0
Pfu	2.0
	60

Incubated 30 min at 72°C

A fraction of non-polished E47⁻ was used for ligation with Fragment 333.

Continued on Page 16

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PROJECT Production of a BamHI fragment of TFL for ligation in E47⁻ Continued From Page

INTRODUCTION

In order to overcome the problems with blunt-end cloning of the 880-bpTFL to get an orientation in the E47⁻, a fragment of 660 bp with a BamHI site in both ends were made by PCR (pfu), and the ends cut with BamHI. E47Θ (see page 15-17) was opened with BamHI ported and de-phosphorylated. After all enzymatic reactions, and purifica the fragment was ligated into E47⁻.

PROCEDURE

4 x 50 µl PFU-pcr reactions was set up (like page 11) The primers used and the following sequence:

5' : 5'- GGG ATC CCC ATG TCT AGG TCT GTG GAG CC-3' 0→

3' : 5'- GGG ATC CCA CAA CTG GGA TAG CCA-3' ←560

The conditions for the PCR was as described page 11.

15µg of E47Θ was cut for 3 hrs with 50 U BamHI.

Fragments and vector was purified using Qia-gen PCR spin columns. Then all the vector was de-phosphorylated using 1U of Shrimp Alkaline Phosphatase vector was then purified, and the concentration of vector and fragment was determined from an DNA mass ladder on a agarose gel.

E47⁻ de	50 ng/µl ~ 21.37 fmol/µl	Ligation			
660	40 ng/µl ~ 182.50 fmol/µl	Vector	4.68	0.47	
		660 fragment	0.78	0.58	
From this, two ligations were		Buffer	4.0	4.0	
setup for 1 hr. incubation at		T4 Ligase	1.0	1.0	
RT. Transformation into electro-		H_2O	7.31	13.55	
competent cells.			20.0	20.0	

Continued on Page 44

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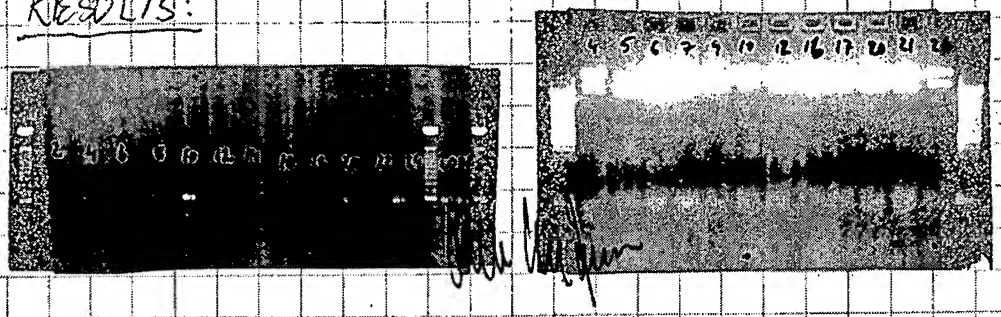
24 colonies were screened by PCR (as described before) using the two primers shown on page 43. From the results, 12 colonies were selected for miniprep and Circle-cutting with XbaI and EcoRI

RESULTS:

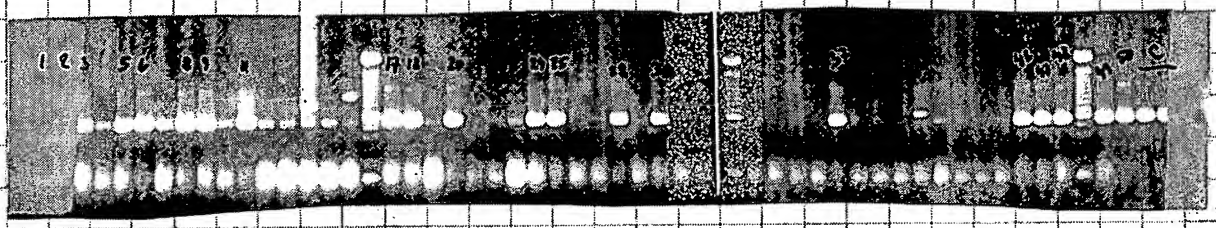


On the basis of the results with the circle cutting only number 10 and 23 were selected

The experiment was repeated, but this time the ligations were done at 12°C for 24 hrs. Transformation into competent cells.

Colony-PCR was performed on 50 randomly selected colonies.

RESULTS:



Following colonies were selected for miniprep and sequencing.

The minipreps were restriction digested with XbaI and SSTI

According to the results some constructs

		Antisense
9		1
18		3
24		8

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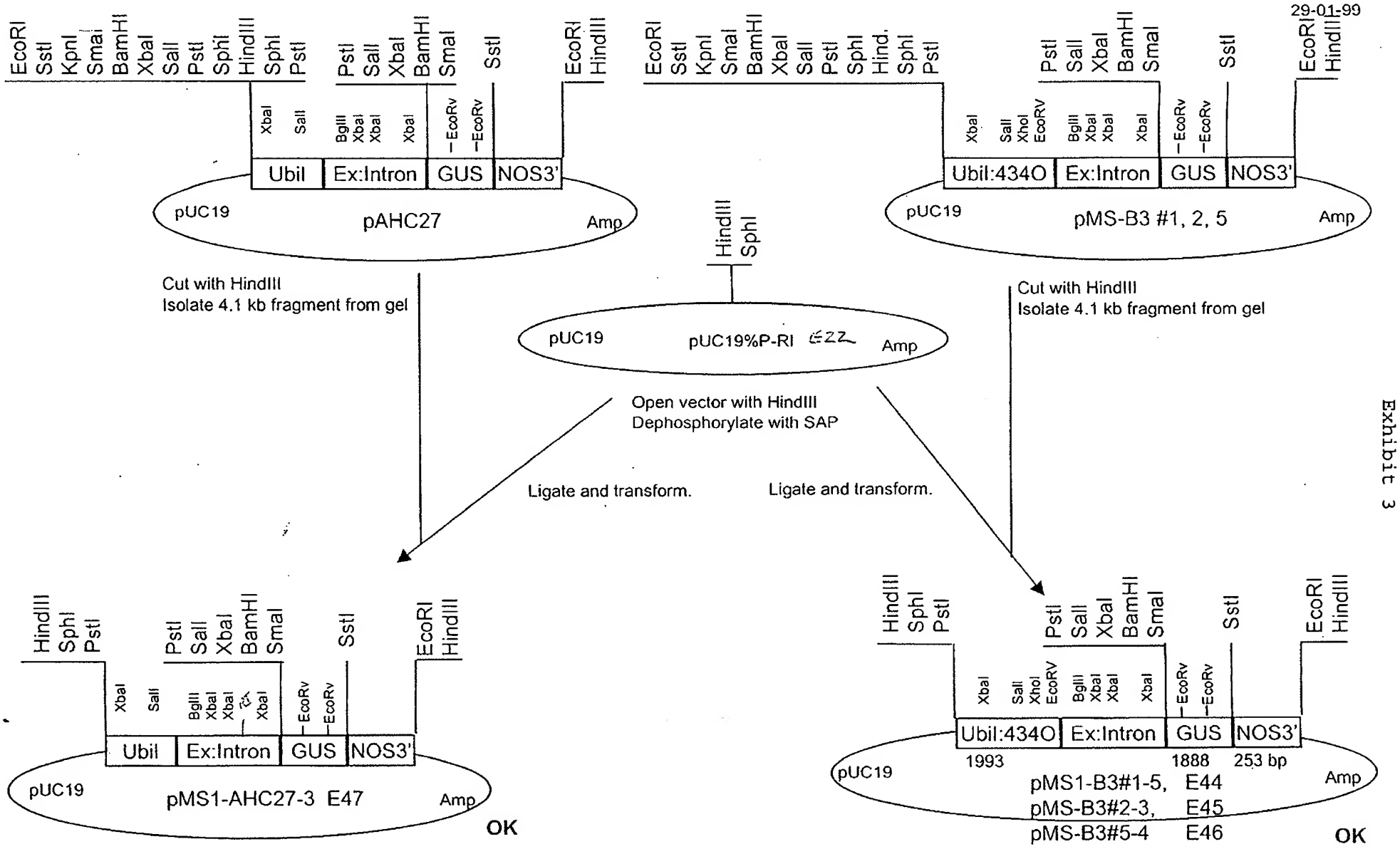

29-01-99

Exhibit 3

B22 Exhibit 4 Ref 2.

Transgenic Research 5, 213–218 (1996)

TECHNICAL NOTE

Ubiquitin promoter-based vectors for high-level expression of selectable and/or screenable marker genes in monocotyledonous plants

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A set of plasmids has been constructed utilizing the promoter, 5′ untranslated exon, and first intron of the maize ubiquitin (*Ubi-1*) gene to drive expression of protein coding sequences of choice. Plasmids containing chimaeric genes for ubiquitin-luciferase (*Ubi-Luc*), ubiquitin-β-glucuronidase (*Ubi-GUS*), and ubiquitin-phosphinothricin acetyl transferase (*Ubi-bar*) have been generated, as well as a construct containing chimaeric genes for both *Ubi-GUS* and *Ubi-bar* in a single plasmid. Another construct was generated to allow cloning of protein coding sequences of choice on *Bam* HI and *Bam* HI–compatible restriction fragments downstream of the *Ubi-1* gene fragment. Because the *Ubi-1* promotor has been shown to be highly active in monocots, these constructs may be useful for generating high-level gene expression of selectable markers to facilitate efficient transformation of monocots, to drive expression of reference reporter genes in studies of gene expression, and to provide expression of biotechnologically important protein products in transgenic plants.

Keywords: gene expression; transgenic monocots; ubiquitin

Introduction

The general availability of strong promoters active in all or most cell types of monocotyledonous plants would be useful in a variety of applications in gene transfer studies with this plant group (McElroy and Brettell, 1994). Although the widely-used cauliflower mosaic virus (CaMV) 35S promoter is active in monocot cells, its relative strength is substantially less than in dicot cells, and it is inactive in some cell types, e.g. pollen (Bruce *et al.*, 1989; Christensen *et al.*, 1992; McElroy and Brettel, 1994). The maize *Adh1* promoter has also been used in monocot transformation studies (Fromm *et al.*, 1990), but its activity appears to be restricted to root and shoot meristems, endosperm, and pollen (Kyozuka *et al.*, 1991). Because of their expected involvement in fundamental processes in all cell types, the genes for rice actin (*Act-1*) (McElroy *et al.*, 1990) and maize ubiquitin (*Ubi-1*) (Christensen *et al.*, 1992) have been investigated as potentially useful alternatives to the CaMV 35S and *Adh1* sequences. Both of these monocot promoters have been shown to be significantly more active than the CaMV 35S promoter in monocot cells (Bruce *et al.*, 1989; McElroy *et al.*, 1990; Christensen *et al.*, 1992; Cornejo *et al.*, 1993; Gallo-Meagher and Irvine, 1993; McElroy and Brettell, 1994) with the *Ubi-1* promoter being somewhat stronger than the *Act-1* promoter where compared directly (Cornejo *et al.*, 1993; Gallo-Meagher and Irvine, 1993; Schledzewski and Mendel, 1994; Wilmink *et al.*, 1995).

Since our initial reports on the use of maize *Ubi-1* promoter constructs in transient (Christensen *et al.*, 1992) and stable (Toki *et al.*, 1992; Uchimiya *et al.*, 1993) cereal transformation studies, we have distributed to a large number of researchers a variety of constructs with the *Ubi-1* promoter fused to a spectrum of selectable and scorable markers. Certain of these constructs or their derivatives have been used successfully in transforming a number of different monocot species (Wilmink *et al.*, 1995), including several cereals (McElroy and Brettell, 1994) and *Lemna* (Rolfe and Tobin, 1991), with reports of transgenic plants having been generated for rice (Cornejo *et al.*, 1993), wheat (Weeks *et al.*, 1993), and

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barley (Wan and Lemaux, 1994). This report presents the structural details of the complete set of these constructs.

Materials and methods

The cloning and sequencing of the maize ubiquitin gene and its promoter have been reported previously (Christensen *et al.*, 1992). All DNA cloning and manipulations reported here were performed according to standard protocols (Ausubel *et al.*, 1989). Restriction endonuclease digestions were carried out according to manufacturers' recommendations. GeneClean (BIO 101, La Jolla, CA, USA) was used to isolate specific restriction fragments from agarose gels. Recovery of DNA fragments was quantified by comparison of ethidium bromide fluorescence of an aliquot of the fragment with known masses of DNA on agarose gels. The *Hin*dIII linker (5′ CAAGCTTG 3′) used in the construction of pAHC27 was obtained from New England Biolabs. DNA ligations and subsequent transformations into competent *Escherichia coli* strain XL1-Blue or HB101 cells and plasmid DNA preparations were carried out using standard protocols (Ausubel *et al.*, 1989). Analysis of DNA sequences was performed using the UWGCG package of programs (Fromm *et al.*, 1990) and DNA Inspector II (Textco, W. Lebanon, NH, USA).

Results

All of the constructs described here were generated by fusing the same 1992 bp *Pst*I fragment from the maize *Ubi-1* gene upstream of the relevant polylinker or marker sequence (Fig. 1). This *Ubi-1 Pst*I fragment contains 899 bp of promoter sequence, 83 bp of 5′ untranslated exon, and 1010 bp of first intron sequence, terminating through reconstitution of the *Pst*I site precisely at the G in the AG dinucleotide of the 3′ splice junction of the intron (Christensen *et al.*, 1992). The nucleotide sequences at the fusion junctions at the 3′ end of the *Ubi-1* DNA are shown for each construct in Fig. 2.

pAHC17

This plasmid is a *Ubi-1* promoter expression vector for *Bam*HI (or *Bam*HI-compatible) cloning of protein coding regions. It contains the *Ubi-1* promoter, 5′ untranslated region and intron upstream of an unique *Bam*HI site (Fig. 1). About 250 bp of nopaline synthase (NOS) 3′ untranslated sequence and polyadenylation signals are located downstream of the *Bam*HI site. The 1992 bp *Pst*I fragment of the *Ubi-1* gene had been previously cloned into the *Pst*I site of M13mp19 for sequencing (Christensen *et al.*, 1992). A *Hin*dIII-*Bam*HI fragment from the replicative form of that clone was isolated and ligated to a 3175 bp *Hin* dIII–*Bam*HI fragment of pMF6 (Goff *et al.*, 1991) containing pUC8 sequence and 250 bp of NOS 3′ polyadenylation sequence adjacent to the *Eco*RI site.

The polylinker sequence is located between the end of the *Ubi-1* intron and the *Bam*HI cloning site and between the *Bam*HI site and the NOS sequence (Fig. 2). Thus, a reporter gene cloned into the *Bam*HI site is flanked by polylinker sequence on both the 5′ and 3′ sides. *Sal*I and *Xba*I sites from the M13mp19 polylinker are upstream of the *Bam*HI site and a *Sal*I and a *Pst*I site from the pUC8 polylinker are on the 3′ side.

pAHC15 and pAHC27 (pUbi-GUS)

These plasmids contain the maize *Ubi-1* promoter, 5′ untranslated region and first intron fused to the coding region of the *E. coli uidA* gene (GUS) (Fig. 1). To produce pAHC15, *Hin*dIII–*Eco*RI fragment of pBI101.2 (Jefferson *et al.*, 1987) containing the *Hin*dIII to *Sma*I region of the pUC19 polylinker, the GUS coding sequence, and 260 bp of the nopaline synthase gene polyadenylation signal was cloned into the *Hin*dIII and *Eco*RI sites of pUC19 (pUC19-GUS-NOS). The 1992 bp *Pst*I fragment of the maize *Ubi-1* gene (Christensen *et al.*, 1992) was cloned into the *Pst*I site of the polylinker sequence upstream of the GUS coding sequence in pUC19-GUS-NOS. The construct contains the *Ubi-1* sequence in an orientation such that transcription will proceed through the ubiquitin 5′ exon, intron and the GUS coding sequence, terminating in the NOS 3′ sequence.

pAHC27 contains the same Ubi-GUS-NOS construct as pACH15 but as a *Hin*dIII fragment cloned into the *Hin*dIII site of pUC19 (Fig. 1). This construct was generated to facilitate the production of pAHC25 (see below). The *Eco*RI site at the 3′ end of the chimeric gene in pAHC15 is not unique as there is an additional *Eco*RI site in the *Ubi-1* intron. However, the *Hin*dIII site at the 5′ end of the chimaeric gene is unique. To allow the entire construct to be removed as one fragment for further subcloning, a *Hin*dIII site was introduced at the 3′ end of the chimaeric gene. This was achieved by partially digesting pAHC15 with *Eco*RI, optimizing the digestion for linear fragments. The *Eco*RI sites were filled in with dNTPs and Klenow fragment of DNA Polymerase and a *Hin*dIII linker (5′ CAAGCTTG 3′; New England Biolabs) was added. Addition of the linker also restored the *Eco*RI site. The DNA was digested with *Hin*dIII to remove excess linker and to cut at the 5′ end of the chimaeric gene. The 4.15 kb *Hin*dIII fragment containing the *Ubi-1 gus* chimaeric gene was gel-purified and subcloned into *Hin*dIII–digested pUC19. The chimaeric gene in the resultant pAHC27 is oriented such that the entire pUC19 polylinker is upstream of the *Ubi-1* promoter (Fig. 1).

pAHC18 – pUbi-LUC

This plasmid contains the *Ubi-1* promoter-5′ exon-first

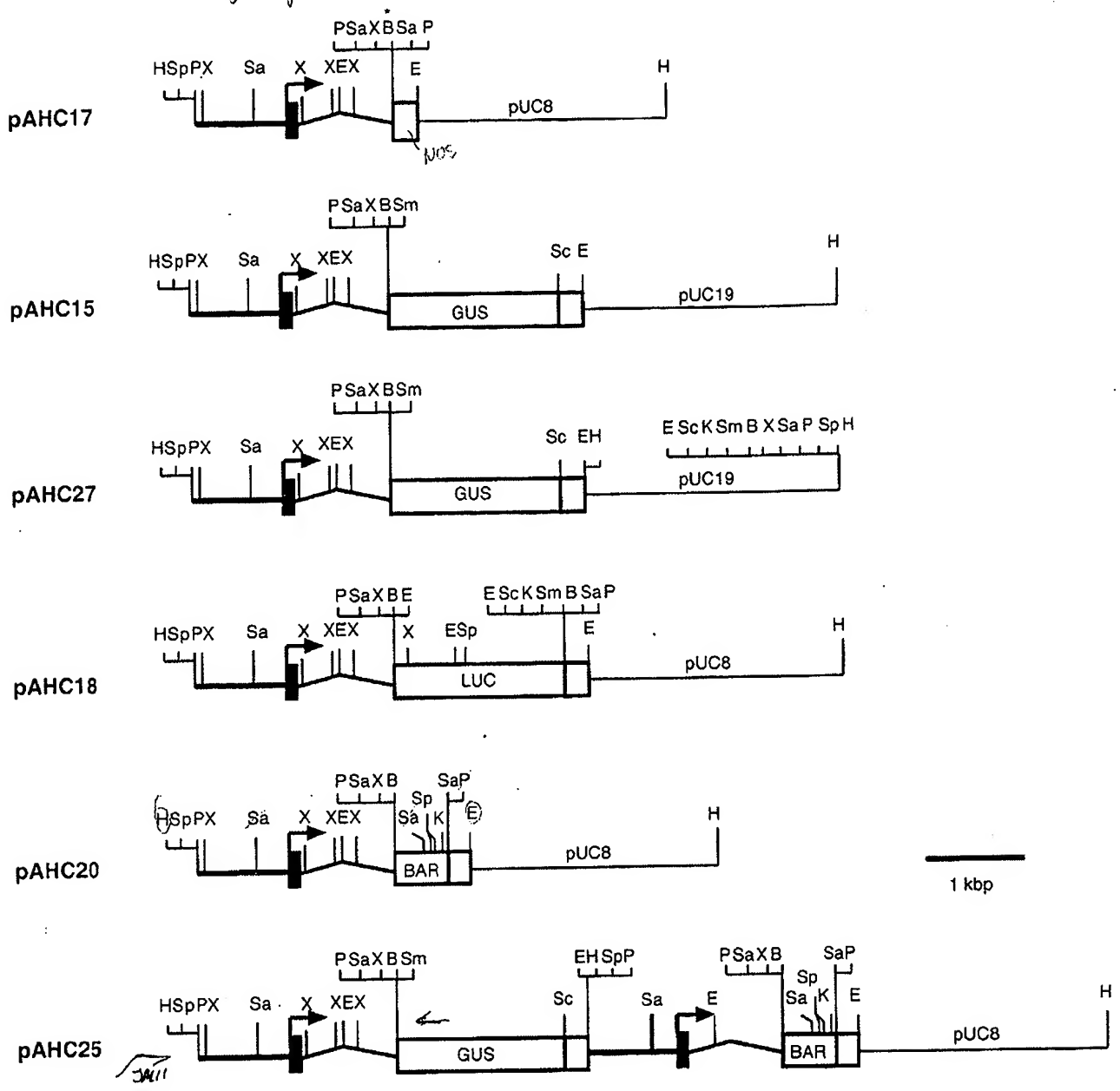

Fig. 1. Schematic diagrams of expression vectors based on maize *Ubi-1* sequences. The relative sizes of the various segments of the linearized plasmids are drawn to scale. Bold straight line, *Ubi-1* promoter sequences; filled box, *Ubi-1* exon; angled line, *Ubi-1* intron; labelled open boxes, reporter gene sequences; blank open box, nopaline synthases 3′ untranslated sequence; thin straight line, pUC8 (pAHC17, 18, 20, and 25) or pUC19 (pAHC15 and 27) sequence. Arrow at the Ubi-1 exon signifies transcription start site and direction. GUS, β-glucuronidase (Jefferson *et al.*, 1987); LUC, firefly luciferase (Ow *et al.*, 1986); BAR, phosphinothricin acetyltransferase (De Block *et al.*, 1987). Restriction sites used in construction of the chimaeric genes and in adjacent polylinker sequences are shown. The *Bam* HI site marked with an asterisk in pAHC17 is an unique site for cloning *Bam* HI or *Bam* HI-compatible fragments. (Note: The *Xba* I sites located in the Ubi-1 intron are subject to methylation interference in *dam* + *E. coli* strains. Also, although the *Eco* RI site in the *Ubi-1* intron upstream of the *gus* sequence in pAHC15 is cleaved efficiently, in both pAHC27 and pAHC25 the corresponding *Eco* RI site is cut inefficiently). B, *Bam* HI; E, *Eco* RI; H, *Hin* dIII; K, *Kpn* I; P, *Pst* I; Sa, *Sal* I; Sc, *Sac* I; Sm, *Sma* I; Sp, *Sph* I; X, *Xba* I.

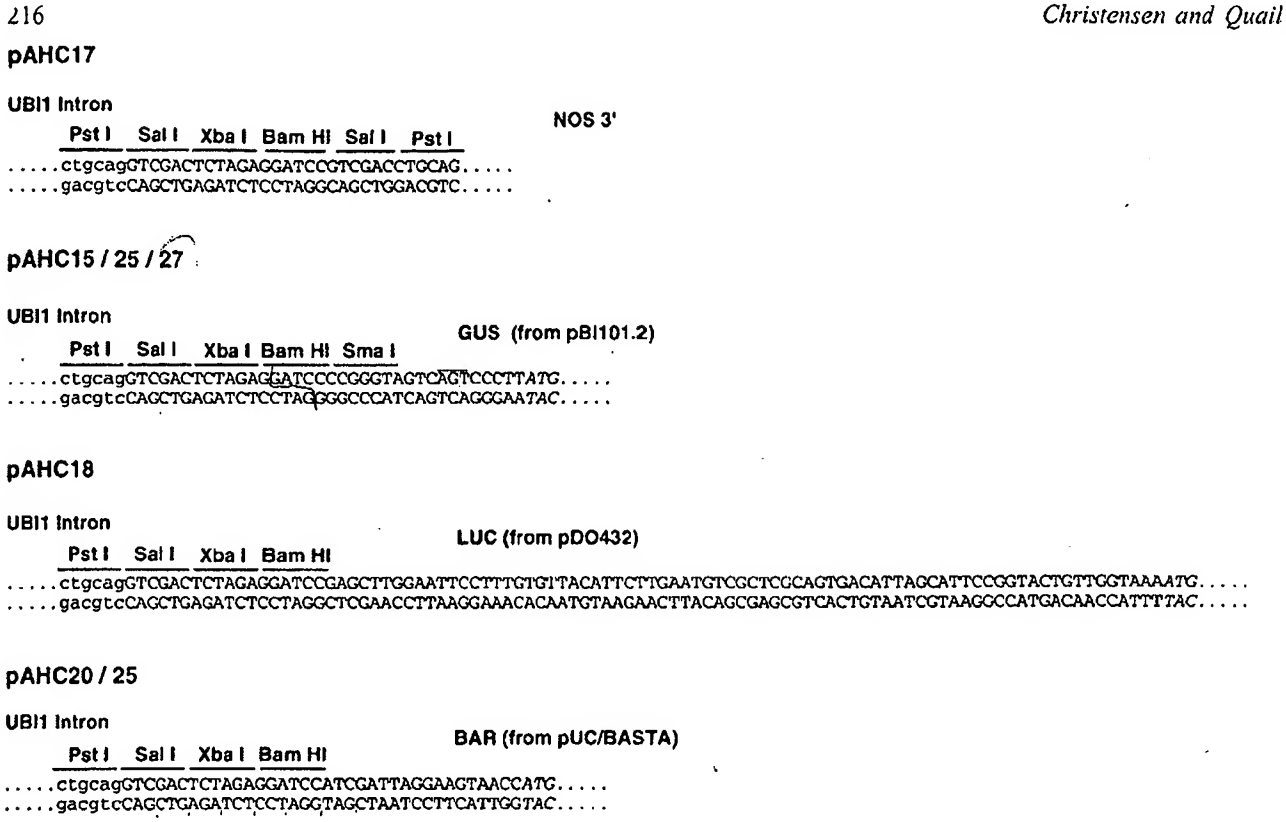

Fig. 2. Nucleotide sequence of the polylinker region comprising the junction between the *Ubi-1* intron and the reporter gene or NOS 3' sequence. The *Ubi-1* intron sequence (lower case) ends with a *Pst* I site containing the 3' splice junction. The reporter gene sequences shown downstream of the polylinker are those upstream of the respective coding sequence and end with the ATG translation start codon (italicized) shown for each.

intron fused to a luciferase (LUC) reporter coding sequence (Fig. 1). An 1892 bp *Bam* HI fragment of pDO432 (Ow *et al.*, 1986) containing 80 nucleotides of 5' untranslated sequence, the luciferase coding region (1649 nucleotides) and 163 bp of 3' untranslated sequence was cloned into the unique *Bam* HI site of pAHC17. This construct contains the luciferase coding sequence in the same orientation as the ubiquitin promoter.

pAHC20 – pUbi-BAR

The Ubi-BAR chimaeric gene in this plasmid provides selection of transformants resistant to Basta™ herbicide (phosphinothricin) (De Block *et al.*, 1987). The Ubi-BAR construct was formed by ligating a 570 bp *Bam* HI–*Bcl* I fragment containing the *bar* gene into the *Bam* HI site of pAHC17. The *bar* gene fragment was excised from a plasmid (pUC8/BASTA) obtained from Dr M. Fromm (Fromm *et al.*, 1990). The resultant pAHC20 plasmid has *bar* in the same orientation as the maize Ubi-1 promoter (Fig. 1). The construct contains 18 bp of sequence between the *Bam* HI site and the translation start codon of the *bar* gene (Fig. 2). The *Bcl* I site is 11 bp downstream of the TGA stop codon.

The unique *Hin* dIII site at the 5' end of the Ubi-1 sequence makes this plasmid very adaptable. This restriction site is suitable for insertion of a second chimaeric gene, such as a scorable marker also driven by a second *Ubi-1* promoter, as detailed below for pAHC25, or for any other desired promoter–gene combination.

pAHC25 – pUbi-GUS/Ubi-BAR

pAHC25 contains both a selectable marker (*bar*) and a scorable marker (GUS), each under the transcriptional control of a separate *Ubi-1* promoter (Fig. 1). The two chimaeric genes were first assembled separately in pAHC20 and pAHC27 and then the double construct was formed. This was achieved by excising the Ubi-GUS-NOS-containing *Hin* dIII fragment from pAHC27 and subcloning it into *Hin* dIII-digested pAHC20. The resultant pAHC25 plasmid has both Ubi-BAR and Ubi-GUS chimaeric genes in the same orientation.

Discussion

The high activity of the maize *Ubi-1* promoter has now been documented in transient and/or stable transformation configurations in a number of monocot systems including rice (Bruce *et al.*, 1989; Toki *et al.*, 1992; Cornejo *et al.*, 1993; Uchimiya *et al.*, 1993; Takimoto *et al.*, 1994), wheat (Taylor *et al.*, 1993; Weeks *et al.*, 1993), barley (Wan and Lemaux, 1994), sugarcane (Gallo-Meagher *et al.*, 1993; Taylor *et al.*, 1993), maize (Christensen *et al.*, 1992; Gallo-Meagher *et al.*, 1993) *Pennisetum* (Taylor *et al.*, 1993), *Panicum* (Taylor *et al.*, 1993) and *Lemna* (Rolfe and Tobin., 1991). Whether or not the high level of expression of selectable marker genes fused to *Ubi-1* actually increases the efficiency of recovery of fertile transgenic plants relative to less active promoters like that from the CaMV 35S gene is yet to be rigorously examined (see Wan and Lemaux, 1994). However, the high level of GUS expression provided by the Ubi-GUS constructs has proven valuable in enabling rapid histochemical screening of transformants for transgene activity (Cornejo *et al.*, 1993).

The original intron present in the 5'-untranslated region of the Ubi-1 gene (Christensen *et al.*, 1992) was retained in all the constructs here because of numerous previous studies showing that introns frequently strongly enhance transgene expression in cereals (Callis *et al.*, 1988; Bruce and Quail, 1990; McElroy *et al.*, 1990; Vasil *et al.*, 1993). The influence of the *Ubi-1* intron has not been tested directly, but there is evidence that this maize sequence is spliced correctly in transgenic rice cells (Toki *et al.*, 1992).

Detailed examination of the spatial and temporal expression patterns of the *Ubi-1* promoter in transgenic plants is yet to be reported. However, initial data with a *Ubi-gus* construct indicate expression in all organs of transgenic rice consistent with a potential for targeting a wide spectrum of cells (Cornejo *et al.*, 1993; Takimoto *et al.*, 1994).

An additional potentially usefull feature of the *Ubi-1* promoter is that it is stress-inducible. Both thermal and mechanical stress have been shown to cause a strong enhancement of the *Ubi-gus* transgene activity in transformed rice (Cornejo *et al.*, 1993; Takimoto *et al.*, 1994). It is possible that this fact may result in stronger expression of selectable marker fusion genes during the early stages of transformation, where recipient cells are exposed to a variety of stresses such as high osmotic pressures, particle bombardment and growth on toxic compounds. A subsequent decrease in expression level is expected upon removal of the selective conditions so that regenerated transgenic plants would presumably not continue to express the marker at high levels when it is no longer needed. The stress-inducibility of the *Ubi-1* promoter might also be useful for driving conditional expression of genes that confer tolerance or resistance to various biotic and abiotic stresses such as pathogen attack, heat and water deficit (Takimoto *et al.*, 1994).

Acknowledgements

We thank W. Bruce and other members of the laboratory for helpful suggestions in the preparation of these plasmid constructs, M. Fromm for suggestions and plasmids, J. Tepperman, D. McElroy and P. Lemaux for comments on the manuscript, and R. Wells for manuscript preparation and editing. This research was supported by USDA-NRICGP no. 92-37301-7678 and USDA CRIS no. 5335-21000-006-00D.

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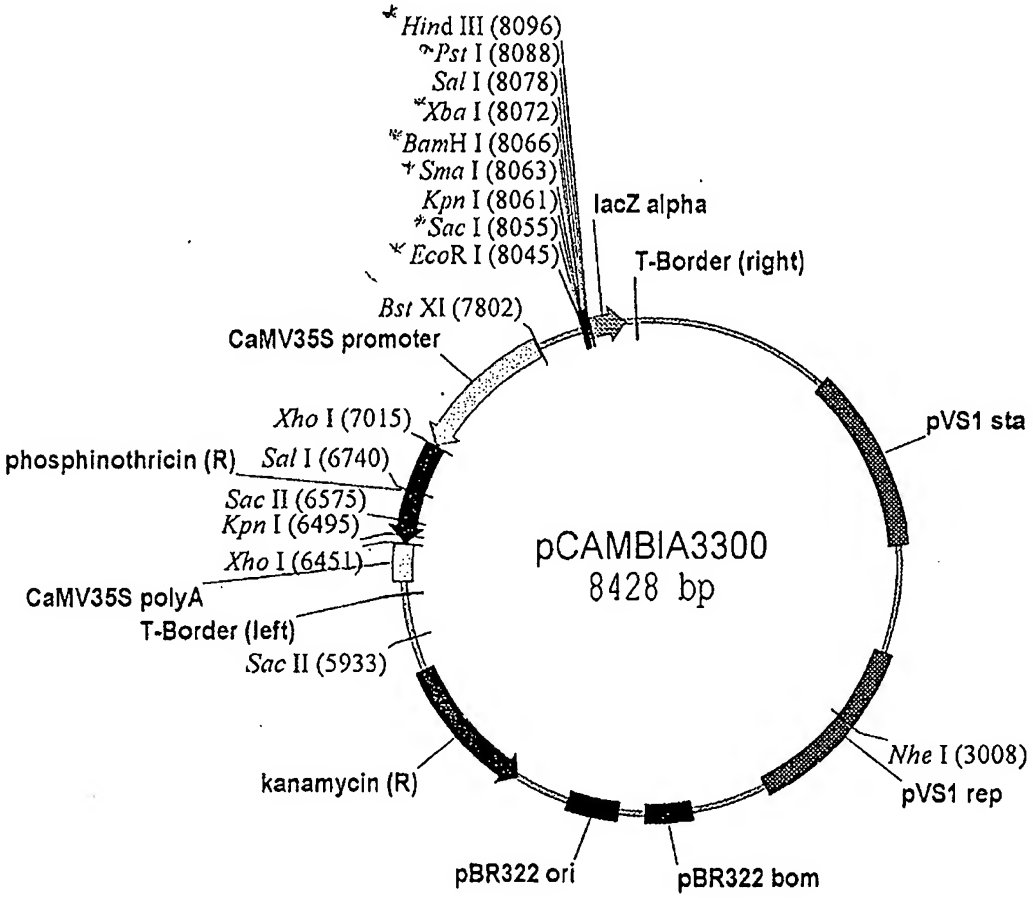
GEN : 1994-3595

TATA: 870 – 876

ATG: 1959

EXON:

DIDIER.THOMAS@AGUK.ZENECA.COM



* Unique.

!! 2× EcoRV sites.

pCAMBIA3300

General Description

DNA Plasmid pCAMBIA3300
PPT resistant pCAMBIA1300 backbone
Currently local object. Original author: Leon Smith
Created: 09/11/97 01:54AM
Last Modified: 09/11/97 01:54AM
length: 8428 bp
storage type: Constructed
form: Circular

Original Author

Leon Smith
EMAIL: lon@cambia.org.au

Comments

Component Fragments

#1: FRAGMENT of pCAMBIA1300

parent position: from 7929 to 6834
length: 7864
molecule position: from 7015 to 6450

Left Terminus

XhoI site #2

Right Terminus

XhoI site #1

#2: FRAGMENT of bar PCR fragment (Complementary)

parent position: from 11 to 574
length: 564
molecule position: from 6451 to 7014

Left Terminus

XhoI site #1

Right Terminus

XhoI site #2

Restriction Map: (no more than 3 sites)

BglII: 0 sites A|GATCT / TCTAG|A

BstEII: G|GTNACC / CCANTG|G

NcoI: C|CATGG / GGTAC|C

SpeI: A|CTAGT / TGATC|A

AvrII: C|CTAGG / GGATC|C

PmlI: CAC|GTG / GTG|CAC

BamHI: 1 site G|GATCC / CCTAG|G

N1: 8066

BstXI: 1 site CCANNNNN|NTGG / GGTNNNNNN|ACC

N1: 7802

EcoRI: 1 site G|AATTC / CTTAA|G

N1: 8045

HindIII: 1 site A|AGCTT / TTCGA|A

N1: 8096

NheI: 1 site GCTAGC / CGATCG
N1: 3008

PstI: 1 site CTGCAG / GACGTC
N1: 8088

SacI: 1 site GAGCTC / CTCGAG
N1: 8055

SmaI: 1 site CCCGGG / GGGCCC
N1: 8063

XbaI: 1 site TCTAGA / AGATCT
N1: 8072

KpnI: 2 sites GGTACC / CCATGG
N1: 6495
N2: 8061

SacII: 2 sites CCGCGG / GGCGCC
N1: 5933
N2: 6575

SalI: 2 sites GTCGAC / CAGCTG
N1: 6740
N2: 8078

XhoI: 2 sites CTCGAG / GAGCTC
N1: 6451
N2: 7015

SphI: 4 sites GCATGC / CGTACG

Functional Map

CDS (3 signals)

kanamycin (R)
Start: 4957 End: 5748 (Complementary)
kanamycin gene amplified from pIG121Hm

bar
Start: 6456 End: 7007 (Complementary)
phosphinothricin acetyltransferase from Streptomyces hygroscopicus ATCC21705

LacZ alpha
Start: 8048 End: 8282
Lac Z alpha fragment

Misc_feature (4 signals)

pVS1 sta
Start: 980 End: 1980 (Complementary)

pBR322 bom
Start: 3983 End: 4243 (Complementary)

T-Border (left)
Start: 6173 End: 6198
Left border repeat from C58 T-DNA

T-Border (right)
Start: 8342 End: 8367
T-DNA repeat

PolyA_signal (1 signal)

CaMV35S polyA

Start: 6248 End: 6450

Promoter_prokaryotic (1 signal)

CaMV35S promoter

Start: 7020 End: 7789 (Complementary)
35S Promoter from CaMV (stop short of BstXI site)

Rep_origin (2 signals)

pVS1 rep

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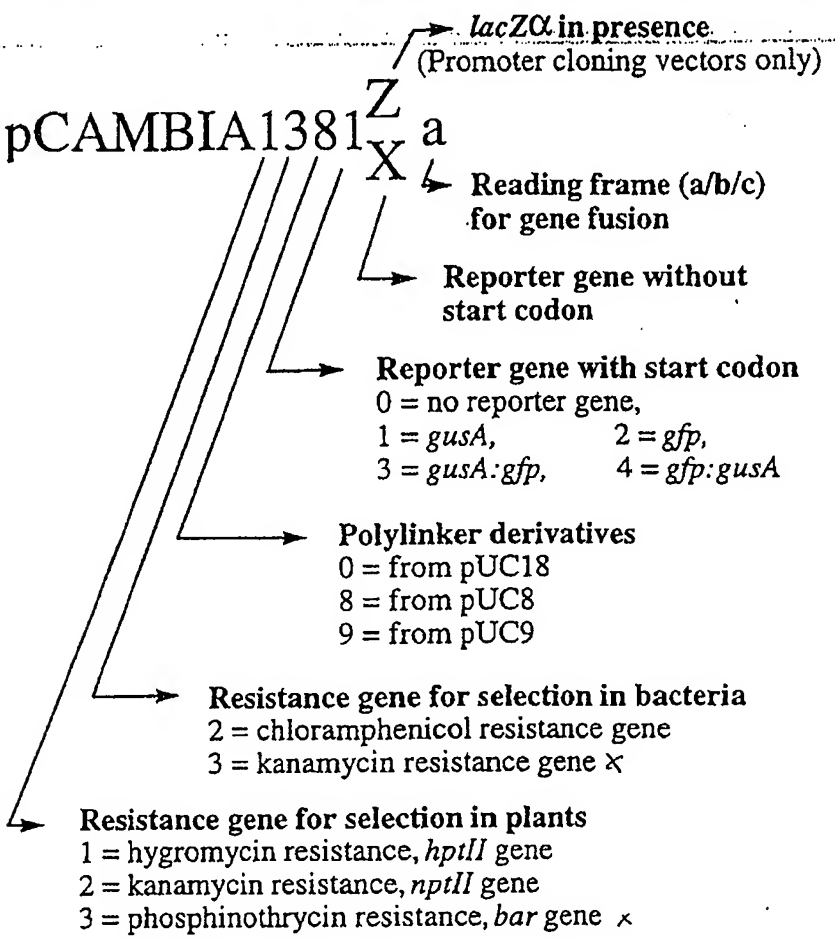
pBR322 ori

Start: 4383 End: 4663 (Complementary)

Annotations

Nomenclature for pCAMBIA Vectors



New Modular Structure of CAMBIA Cassettes

```
                                        Kozak consensus
                                Ter  S/D    Initiator
   BamHI  SmaI      BseRI       NcoI        BglII      SpeI
5'-GGATCCCCCGGGTAAGAGGAGTCCACC ATG GTA GAT CTG ACT AGT   YOU RFA VOU
                    ***           M   V   D   L   T   S     X   X   X

                                Hexa-Histidine Tag          Ter
                        NheI                        PmlI          BstEII
   RIT EGE NE! GCT AGC CAC CAC CAC CAC CAC CAC GTG TGAATTGGTGACC-3'
     X   X   X   A   S   H   H   H   H   H   H   V  ***
```

 CAMBIA GPO Box3200, Canberra ACT 2601, Australia
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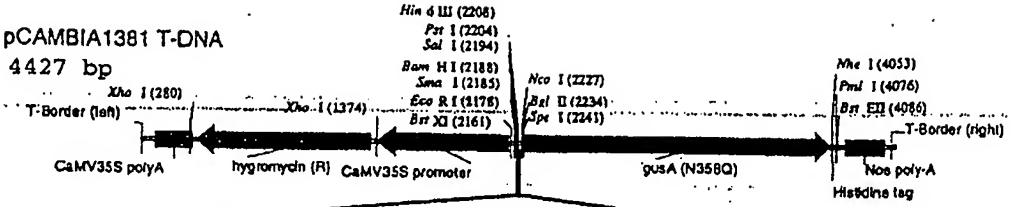

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pCAMBIA1381Xb - reading frame +2
pUC8MCS HindIII Eco47III BglII SpeI gusA
A AGC TTC AGC GCT GTA GAT CTG ACT AGT TTA

pCAMBIA1381Xc - reading frame +3
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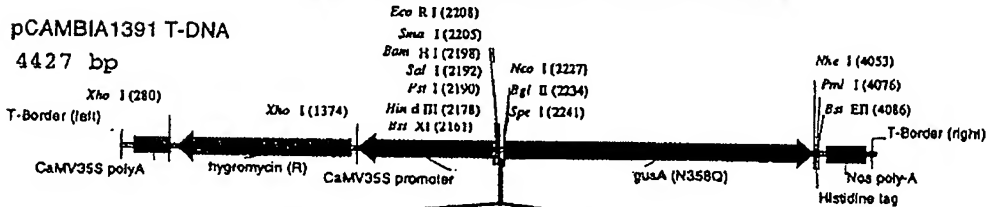

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G AAT TCC AGC GCT GTA GAT CTG ACT AGT TTA

pCAMBIA1391Xc - reading frame +3
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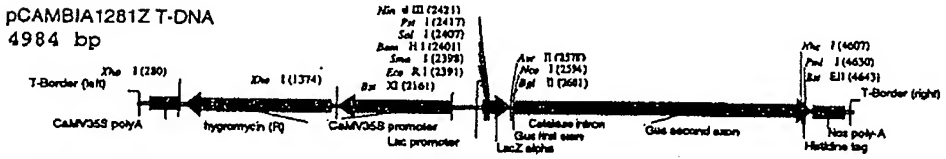

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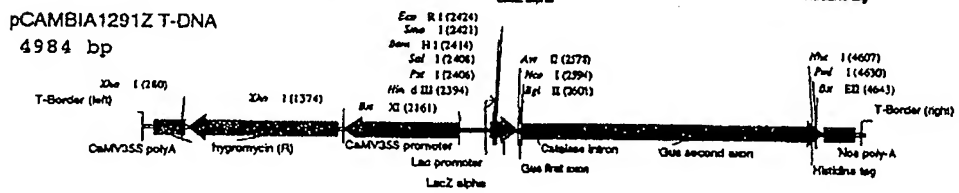

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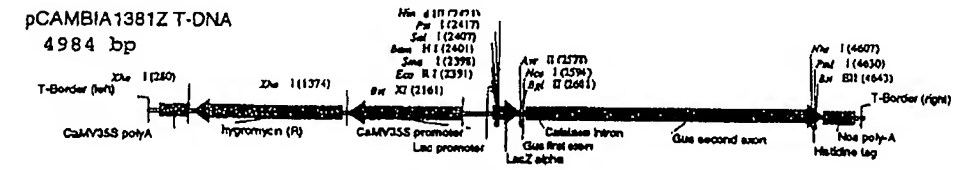

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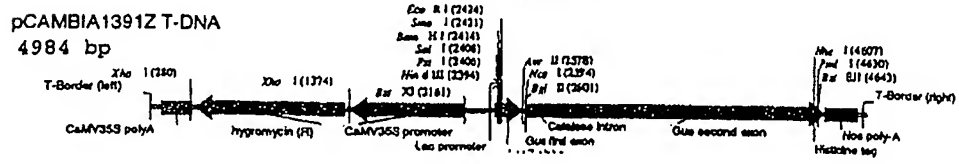

CAMBIA GPO Box3200, Canberra ACT 2601, Australia
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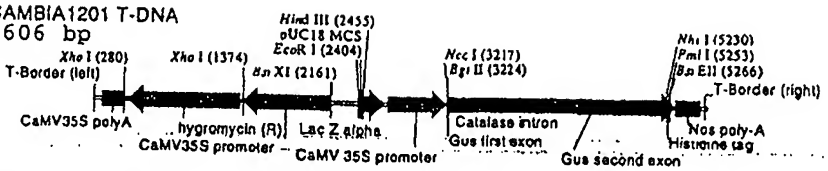

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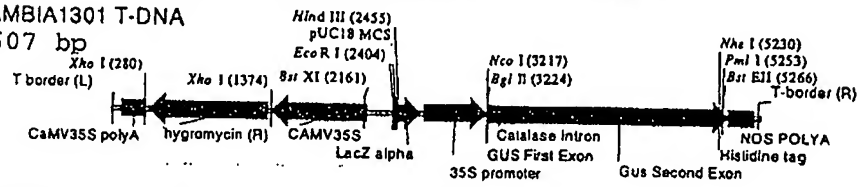

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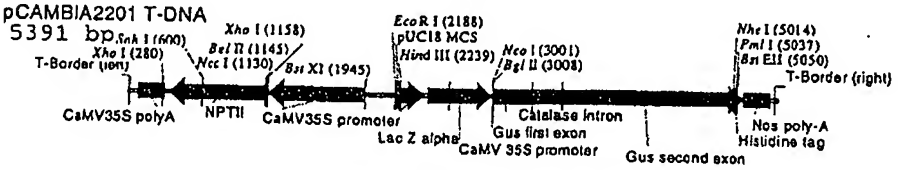

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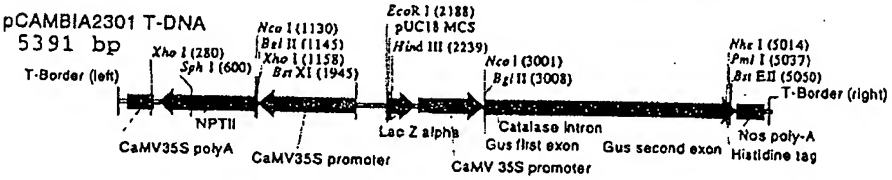

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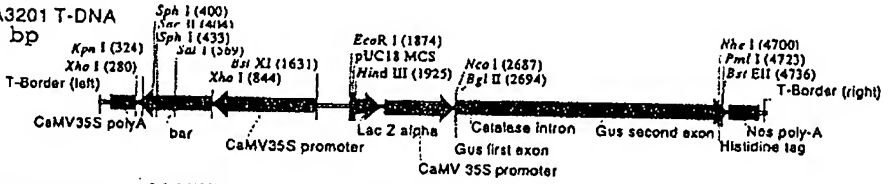

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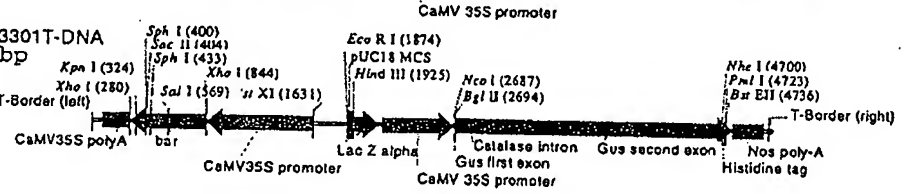

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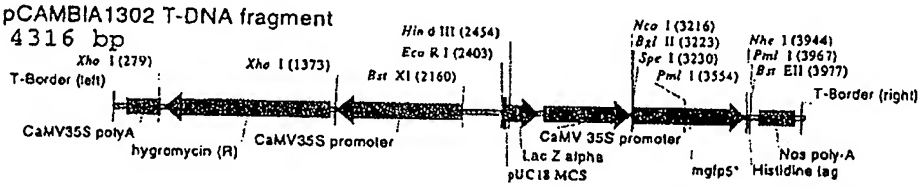

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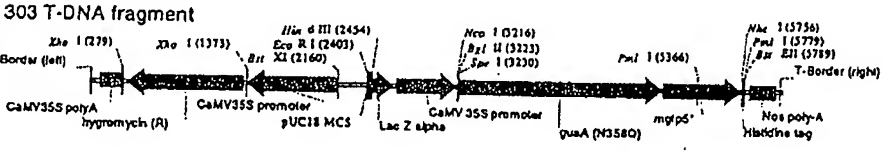

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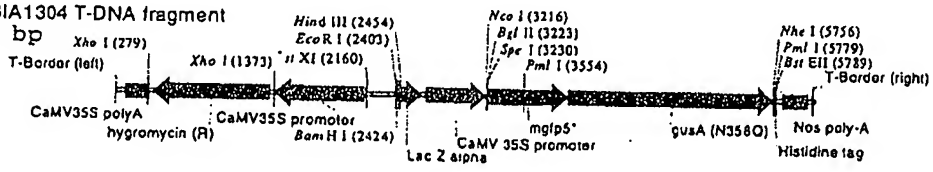

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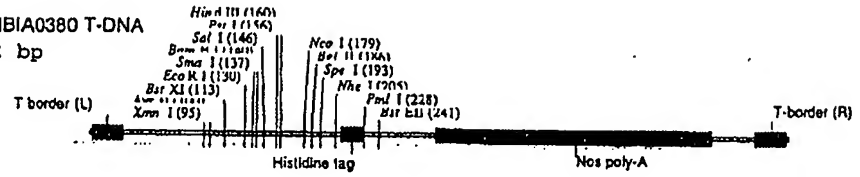

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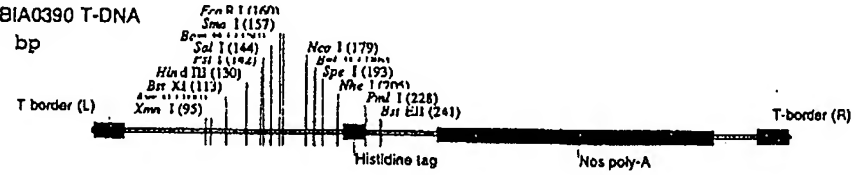

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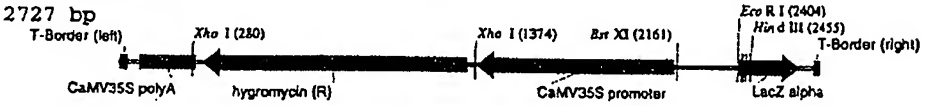

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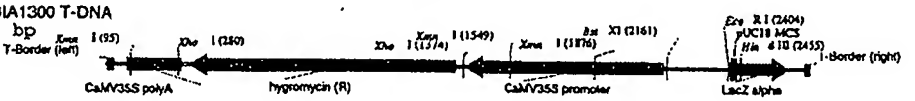

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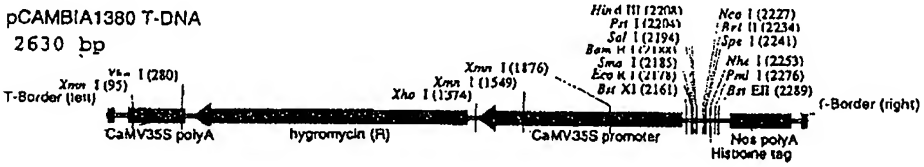

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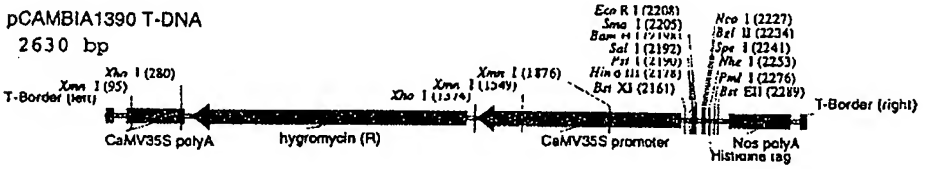

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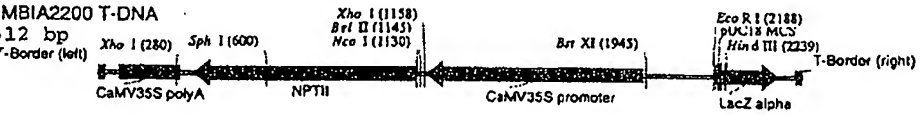

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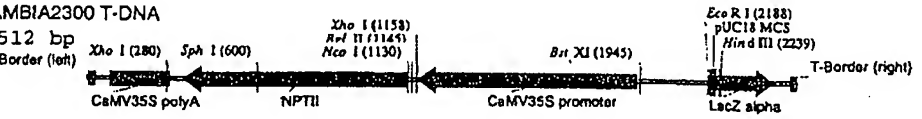

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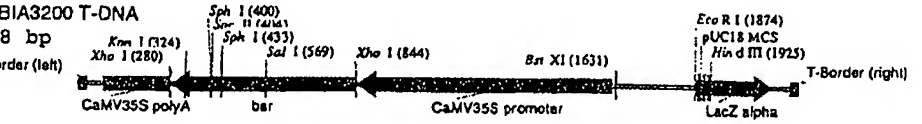
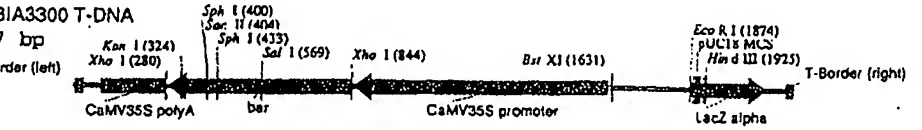
2198 bp



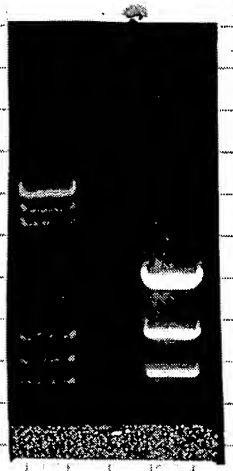
pCAMBIA3300 T-DNA
2197 bp

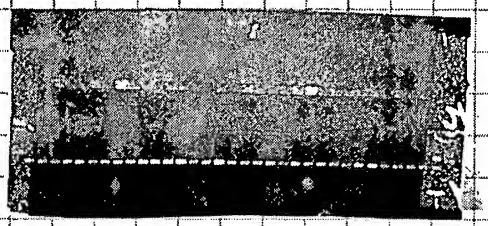


 CAMBIA GPO Box3200, Canberra ACT 2601, Australia
cambia@cambia.org.au
ph. 61 2 6246 5302 fax 61 2 6246 5303

RESULTS:

Release of cassette PCR pUMBin3300 Colonie-PCR

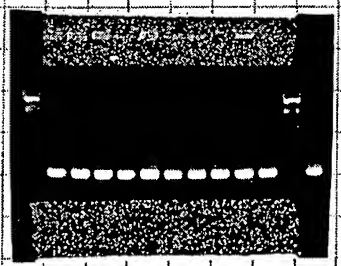


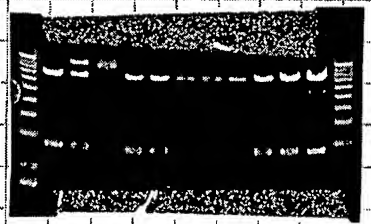


HindIII + EcoRI dig. SacI dig.

Digestion with HindIII + EcoRI and SacI alone gives the right sizes (8700 bp and 2600 bp)

The plasmid was transformed into M. tuberculosis 6UB101 by electroporation insert was verified by Colonie-PCR, and mini prep cultures were made from pos. MM prep was cut with HindIII - EcoRI to release cassette





All colonies positive and match the restriction pattern of plasmid control.

Continued on Page 16

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Date

To complement tfl1 - mutants in Arabidopsis, one mutant line was transformed with the GV3101 containing the pCAMBIA 3300 LpTFL1
This was done according to the 'floral-dip' method described below

Transformation procedure

Results:

Of all seeds harvested and sown out, 20 showed to be basta resistant. PCR on leaf material was performed for the verification of the presence of UBI:LpTFL1

Simplified Arabidopsis Transformation Protocol
(Brief version for those who are familiar with the method)

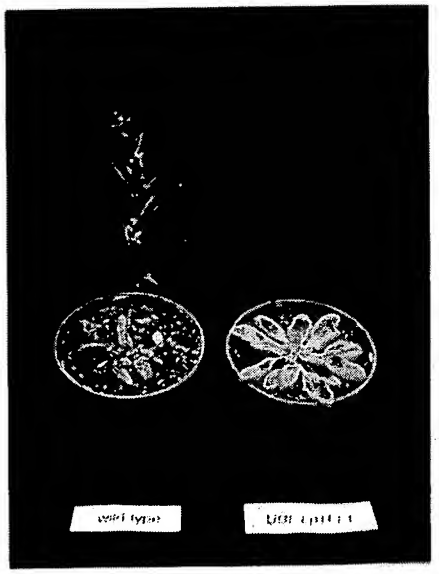
Steve Clough and Andrew Bent, University of Illinois at Urbana-Champaign.
Our present protocol (Clough and Bent, 1998; modified from Bechtold et al. 1993) is extremely simple. We have found that the MS salts, hormone, etc. make no difference, that OD of bacteria doesn't make much of a difference, that vacuum doesn't even make much of a difference as long as you have a decent amount of surfactant present. Plant health is still a major factor - healthy fecund plants make a big difference! With this method you should be able to achieve transformation rates above 1% (one transformant for every 100 seed harvested from Agrobacterium-treated plants).

1. Grow healthy Arabidopsis plants until they are flowering. Grow under long days in pots in soil covered with bridal veil, window screen or cheesecloth.
2. *(optional)* Clip first bolts to encourage proliferation of many secondary bolts. Plants will be ready roughly 4-6 days after clipping. Clipping can be repeated to delay plants. Optimal plants have many immature flower clusters and not many fertilized siliques, although a range of plant stages can be successfully transformed.
3. Prepare *Agrobacterium tumefaciens* strain carrying gene of interest on a binary vector. Grow a large liquid culture @ 25°C in LB with antibiotics to select for the binary plasmid, or grow in other media. You can use mid-log cells or a recently stationary culture.
4. Spin down Agrobacterium, resuspend to OD_{600} = 0.8 (can be higher or lower) in 5% Sucrose solution (if made fresh, no need to autoclave). You will need 100-200 ml for each two or three small pots to be dipped, or 400-500 ml for each two or three 3.5" (9cm) pots.
5. Before dipping, add Silwet L-77 to a concentration of 0.05% (500 ul/L) and mix well. If there are problems with L-77 toxicity, use 0.02% or as low as 0.005%.
6. Dip above-ground parts of plant in Agrobacterium solution for 2 to 3 seconds, with gentle agitation. You should then see a film of liquid coating plant. Some investigators dip inflorescence only, while others also dip rosette to hit the shorter axillary inflorescences.
7. Place dipped plants under a dome or cover for 16 to 24 hours to maintain high humidity (plants can be laid on their side if necessary). Do not expose to excessive sunlight (or under dome can get hot).
8. Water and grow plants normally, tying up loose bolts with wax paper, tape, stakes, twist-ties, or other means. Stop watering as seeds become mature.
9. Harvest dry seed. Transformants are usually all independent, but are guaranteed to be independent if they come off of separate plants.
10. Select for transformants using antibiotic or herbicide selectable marker. For example: vapor-phase sterilize and plate 40 mg ~ 2000 seed (resuspended in 4 ml 0.1% agarose) on 0.5X MS-0.8% tissue culture Agar plates with 50 ug/ml Kanamycin, cold treat for 2 days, and grow under continuous light (50-100 microEinsteins) for 7-10 days.
11. Transplant putative transformants to soil. Grow, test, and use!

For higher rates of transformation, plants may be dipped two or three times at seven day intervals. We suggest one dip two days after clipping, and a second dip one week later. Do not dip less than 6 days apart.

References:

Bechtold, N., Ellis, J., and Pelletier, G. (1993). *In planta Agrobacterium*-mediated gene transfer by infiltration of adult *Arabidopsis thaliana* plants. C. R. Acad. Sci. Paris, Life Sciences 316:1194-1199.
Clough SJ and Bent AF, 1998. Floral dip: a simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana. Plant J 16:735-43
Additional commentary can be found by searching the Arabidopsis newsgroup archives: http://genome-www.stanford.edu/cgi-bin/biosci/arabidopsis
NOTE: Obtain proper approval for transformation work from institutional authorities. Autoclave and properly dispose of all materials.



Wild type and UBI:LpTFL1 transformant

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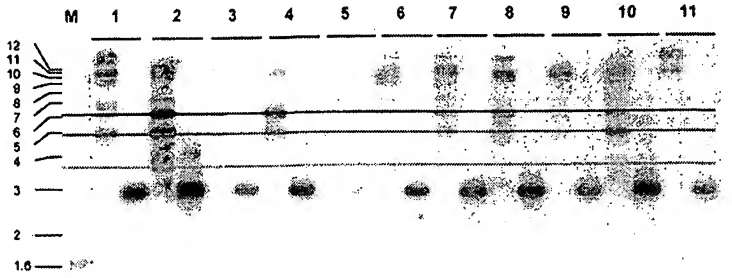
An autoradiogram is shown to the ~~left~~ left. Numbers refer to these line. WT = wild type P = Plasmid

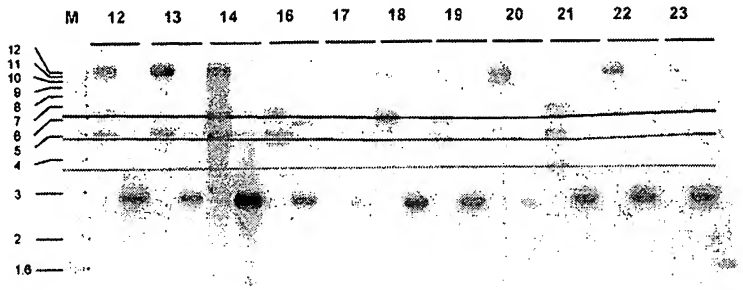
Dark blue line indicate size of head to head tandem repeats

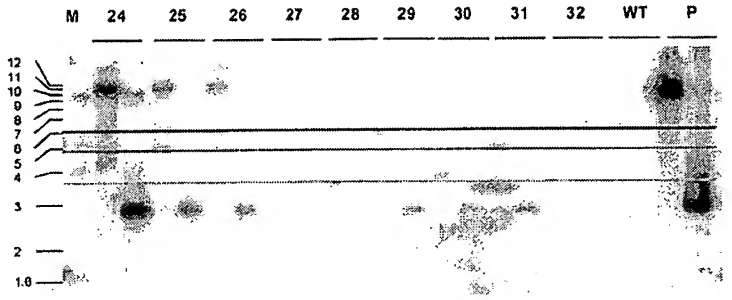
Blue line indicate size of head to tail tandem repeats

Pale blue indicate size of tail to tail (but these is outside the probe area)

Based on these results the lines can be grouped according to the kind of repeats.

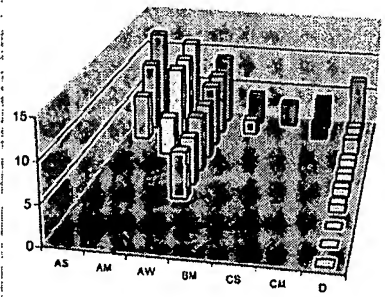






No:	Head to tail	Head to head	Tail to tail	No of bands	Copies	Group
1	1	1	1	7	10	AM
2	1	1	1	9	12	AS
3	0	0	0	1	1	D
4	1	1	1	3	6	AS
5	0	0	0	1	1	D
6	0	0	0	7	7	D
7	1	1	1	8	11	AM
8	1	1	1	7	10	AM
9	1	1	1	3	6	AW
10	1	1	1	7	10	AM
11	0	0	0	3	3	D
12	1	1	1	4	7	AW
13	1	0	0	3	4	BM
14	1	1	1	6	9	AS
15						
16	1	1	1	2	5	AM
17	0	0	0	1	1	D
18	0	1	0	2	3	CS
19	1	1	1	3	6	AW
20	0	0	0	2	2	D
21	1	1	1	5	8	AW
22	0	1	0	3	4	CM
23	1	1	1	2	5	AW
24	1	1	1	6	9	AW
25	1	1	1	4	7	AW
26	1	1	1	5	8	AW
27	0	0	0	0	0	D
28	0	0	0	1	1	D
29	0	1	0	1	2	CM
30	0	0	0	1	1	D
31	1	0	0	1	2	BM
32	0	0	0	0	0	D
33						
0	0	0	0	0	0	0

Copy numbers of *LpTFL1* in transgenic *Arabidopsis*



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21.08.00 — 20/12/00

PROJECT Transformation of L perenne with Sense LpTFL

Notebook No. 3177-C

Continued From Page

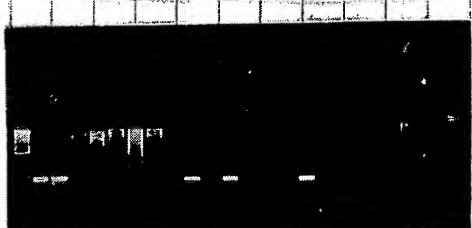
Introduction

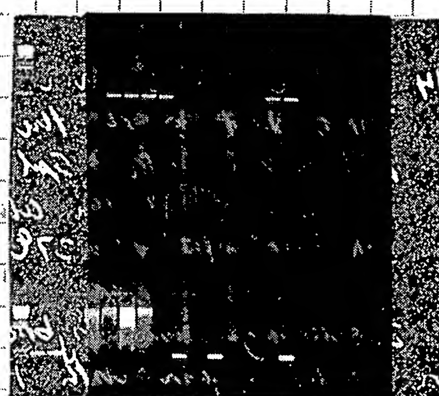
The construct with the Sense orientated LpTFL in the p-BCH27-3 vector was transformed into L perenne by shot-gun gene transfer. Suspension colonies were plated and growing calli on selection were selected for PCR-check of GOI.

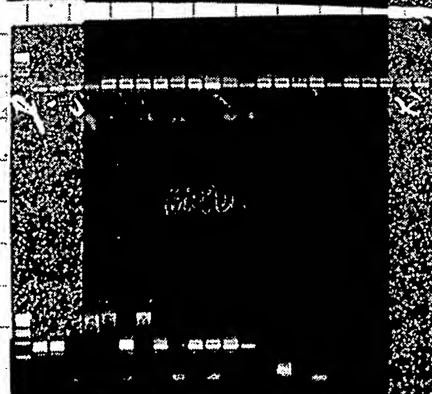
PROCEDURE

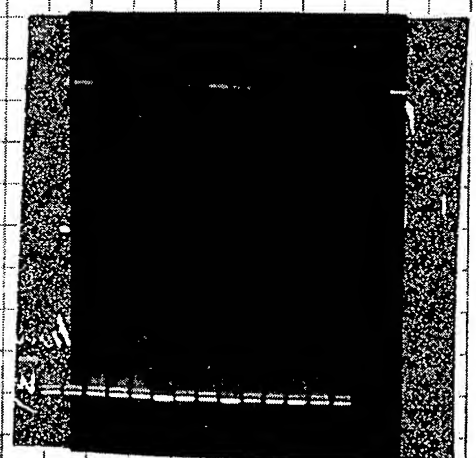
The transformation and growth of putative transformed calli was performed at DLF Trifolium. 58 independent calli were tested for integration of plasmid. Conditions for the PCR reaction were optimized by Anne-Mette Storm Petersen, who also performed the test. As primers were used one from the Ubi-intron and a 3' LpTFL primer. GAPDH primers were used as positive control.

Results:









28 out of 58 putative transformed calli clones contained the sense LpTFL construct as confirmed by this PCR.

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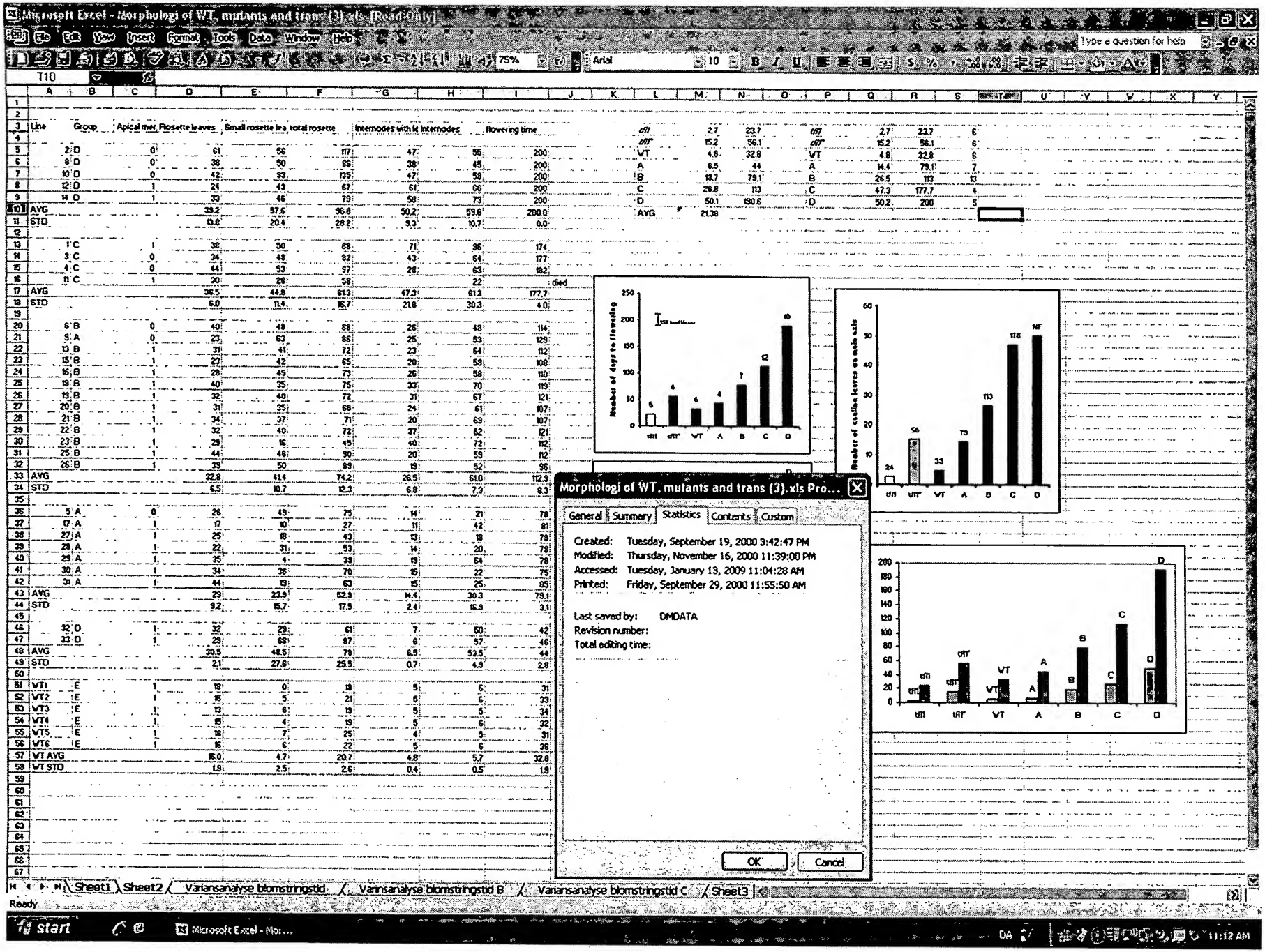
01.02.00 Signed Date Signed 20/12-00 Date



Best Available Copy

Exhibit 11

Exhibit 12

RISØ

Forskningscenter Risø Afdeling for Plantebiologi og Biogeokemi

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December, 22, 2000

Dr. Richard M. Amasino
Department of Biochemistry
University of Wisconsin
433 Babcock Drive
Madison, Wi 53707-1544

Dear Dr. Richard Amasino:

Thank you for reviewing our manuscript so rapidly and thoroughly, and for your helpful remarks on the contents. Based on the reviewers comments we have tried both to clarify some of the unclear results and to specify some of the misleading terms.

The reviewers comments:

Comment 1. Novel aspects or pleiotropic effects of LpTFL1 function compared to TFL1 as revealed by trichome distribution.

We are aware that some of the morphological traits associated with overexpressing *LpTFL1* in *Arabidopsis* can be due to pleiotropic effects of the inserted construct. However, regarding trichome distribution, which is considered as a marker for juvenility in *Arabidopsis*, we do not regard our observations as revealing a new role of LpTFL1, especially because Telfer *et al.*, (1997) who showed the correlation between juvinility and trichome destribution, actually based their conclusions in part on the trichome distribution seen in *tfl1* mutants. In the manuscript we have now stressed this point of view (page 10, parag. 2, line 21 and page 15, parag 2, line 23).

Comment 2. Comparison of *LpTFL1* expression with the expression of homologs in other species.

We would indeed have liked to include an *in situ* study of *LpTFL1* expression in the paper to strengthen our conclusions, however the technique has not yet been established in our lab. We are aware that RT-PCR experiments on *TFL1*-like genes have been reported from tobacco (*CET1/CET6)* and *Antirrhinum*, although the significance from the latter was never confirmed. We have now cited these results on page 9, paragraph 2, line 7

We do not want to draw two different conclusions on *LpTFL1*expression, and as pointed out by the reviewer RT-PCR does not show the location of *LpTFL1* expression within the apex and inflorescence. We realize that the usage of the terms meristem and apex has not been consistent throughout the article, and this might confuse and lead to conflicting interpretations. For this reason we have changed the use of 'ryegrass meristem' to 'ryegrass apex' (which includes the SAM and the axillary meristems in the ridges).

It has been changed in the following paragraphs (in the revised manuscript):

Page 5, paragraph 2, line 23
Page 8, paragraph 3, line 30
Page 9, paragraph 1, line 2
Page 9, paragraph 2, line 10
Page 9, paragraph 2, line 12
Page 14, paragraph 3, line 27
Page 16, paragraph 2, line 13
Page 16, paragraph 2, line 17
Page 17, paragraph 2, line 15

Comment 3. *LpTFL1* Promoter analysis in *Arabidopsis*

Concerning the GUS expression we find that GUS-expression is detected in the axillary meristems of rosette- and cauline leaves, and thus the term 'around the axils of' used in the original manuscript is misleading and has been replaced by 'in the axillary meristem of' (page 12, paragraph 2, line 10, 14, and 18; page 16, paragraph 2, line 18, 20, and 24). New results on T2 generation of *LpTFL1*::GUS plants have been obtained since the first submission. These results confirm the observed GUS activity pattern in the primary transformants and they furthermore show that GUS expression is restricted to the axillary meristems of rosette leaves and the 3-5 lowest cauline leaves which were initiated during the vegetative phase. With a few exceptions, this pattern shows similarities both to the expression of *TFL1*in *Arabidopsis* and the *CET2/CET4* genes in tobacco. Therefore, instead of stating that 'this pattern is an exact reproduction of the expression pattern of the *CET2/CET4* genes in tobacco' we have modified the interpretation by saying: 'The axillary expression of *LpTFL1*::GUS is reminiscent of *CET2/CET4*...'. (page 16 parag. 2). In the same paragraph we also address the need for confirmation of a *LFY*-like effect on *LpTFL1* in ryegrass similar to *NFL* in tobacco. The importance of the regulatory elements driving *LpTFL1* expression is also discussed here and briefly mentioned in the 'results' (page 8, parag. 2, line 23)

Details:

Fig 1A details in the picture has been specified
Fig 1B legend. The term 'edgewise' has been replaced by 'alternately'
Fig 1C Enlargement of floret is described
Fig 6 details in the picture has been specified

Page 5 parag 2. '...lateral meristems. These *become* the leaf primordia' instead of '...These *are* the leaf primordia'.

Page 5, parag 2. Competence. The use of 'competence' in this context is not correct, so the sentence was changed to: 'This morphological pattern does not change until the apex has been induced to flower by...'

Page 8 parag 2. DNA blot analysis suggests there are two *TFL1*-like genes in ryegrass –'maximum' is deleted. Since the blot was performed at medium stringency we believe it is rather unlikley that more that two genes are present.

Page 9 parag 2. *LpTFL1* message was lower in fully matured inflorescences compared to...this refers to the RNase protection assay. Therefore we have added that this is after 42 days sec. induction, and in Fig 3A we have changed the term 'Flower' to 'mature inflorescence'

Page 10 parag. 2. 'The development of floral organs in the axils of cauline leaves' is changed to ' The development of coflorescences with developing flowers in the axils of cauline leaves'

Page 12 Sensitivity of GUS detection. We agree that RT-PCR could reveal whether GUS message is completely absent in other tissues than where it is visibly detected, but a potential low expression would not change the interpretations of our results.

Page 16/17 '...*LpTFL1* ... to promote lateral branching of the main axis and the spikelets. In this way a maximum number of florets are produced' is changed to '...*LpTFL1*... to promote lateral branching of the main axis. In this way a maximum number of spikelets are produced'.

Concerning your comments on the reproducibility of the vernalization effect on levels of *LpTFL1* mRNA, the PCR reactions were repeated three times independently, but the RT reaction was only performed once due to the limited amount of apex tissue. We have indicated in the Discussion that a more refined dissection of this expression pattern needs to be performed in order to draw any substantial conclusions (page 15, parag 1, line 12), and in relation with this we have re-evaluated the term 'significantly' and removed it.

All the changes have been highlighted (underlined) in the revised manuscript. We confirm that we pay the charges for printing color figures.

We hope that the changes made in the revised manuscript are satisfactory, so that we can make the deadline of December 27th, for the special grass issue.

We are looking forward to hearing from you.

Merry Christmas!

Sincerely,

Klaus Salchert

Running Head:

Lolium *CEN/TFL1*-like gene.

Key words: *Lolium perenne*, flower development, meristem identity, plant architecture, *TERMINAL FLOWER1*, *CENTRORADIALIS*, Phosphatidylethanolamine-binding proteins, *Arabidopsis thaliana*

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A *TERMINAL FLOWER1*-like Gene From Perennial Ryegrass Involved in Floral Transition and Axillary Meristem Identity.

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ABSTRACT

Control of flowering and the regulation of plant architecture have been thoroughly investigated in a number of well studied dicot plants such as *Arabidopsis, Antirrhinum*, and tobacco. However in many important monocot seed crops, molecular information on plant reproduction is still limited. In order to investigate the regulation of meristem identity and the control of floral transition in perennial ryegrass (*Lolium perenne* L.), we isolated a ryegrass *TERMINAL FLOWER1*-like gene, *LpTFL1*, and characterized it for its function in ryegrass flower development. Perennial ryegrass requires a cold treatment of at least 12 weeks in order to induce flowering. During this period, levels of *LpTFL1* message in the ryegrass apex is decreased. However, upon subsequent induction with elevated temperatures and long day photoperiods, *LpTFL1* message levels increased and reached a maximum, when the ryegrass apex has formed visible spikelets. *Arabidopsis* plants overexpressing *LpTFL1* were significantly delayed in flowering and exhibited dramatic changes in architecture such as extensive lateral branching, increased growth of all vegetative organs, and a highly increased trichome production. Furthermore, overexpression of *LpTFL1* was able to complement the phenotype of the severe *tfl1-14* mutant of *Arabidopsis*. Analysis of the *LpTFL1* promoter fused to the *UidA* gene in *Arabidopsis* revealed that the promoter is active in axillary meristems but not the apical meristem. We therefore suggest, that *LpTFL1* is a repressor of flowering and a controller of axillary meristem identity in ryegrass.

INTRODUCTION

The life cycle of flowering plants in general can be divided in to three growth phases: vegetative (V), inflorescence (I), and floral (F) (Poethig 1990). In the vegetative phase the shoot apical meristem (SAM) generates leaves that later will ensure the resources necessary to produce fertile offspring. Upon receiving the appropriate environmental and developmental signals, the plant switches to floral, or reproductive, growth and the SAM enters the inflorescence phase (I_1) and gives rise to an inflorescence with flower primordia. During this phase the fate of the SAM and the secondary shoots that arise in the axils of the leaves is determined by a set of meristem identity genes, some of which prevent and some of which promote the development of floral meristems. Once established, the plant enters the late inflorescence phase (I_2) where the floral organs are produced. Two basic types of inflorescences have been identified in plants: determinate and indeterminate (Weberling, 1989). In determinate species the SAM eventually produces floral organs and the production of meristems is terminated with a flower. The SAM of indeterminate species is not converted to a floral identity and will therefore only produce floral meristems from its periphery, resulting in a continuous growth pattern. The regulation of meristem identity and plant architecture has been investigated in a number of dicotyledoneous plants including *Arabidopsis*, *Antirrhinum,* tomato, and tobacco. However, in important seed crops such as wheat, barley, rice, forage grasses, and other monocotyledoneous plants, information on how meristem determinacy is controlled is still limited. Therefore, we have undertaken a molecular investigation of the regulation of meristem identity and the control of floral transition in perennial ryegrass (*Lolium perenne* L.), a cool-season perennial forage grass native to Europe, temperate Asia and North Africa.

In terms of plant development, the aerial parts of ryegrass are produced by the apex positioned on the base plate a few millimeters above the ground and surrounded by developing leaves (Figure 1A). During vegetative growth, the apical meristem generates lateral meristems initially recognized as semicircular ridges along the main axis. These become the leaf primordia. This morphological pattern does not change until the meristem has achieved competence to respond to flowering signals, e.g., elevated temperatures and increasing day length. The minimal requirement for flower induction in perennial ryegrass is a vernalization period of 12-14 weeks below 5°C followed by secondary induction with long day photoperiods (LD, 16 hrs light, 8 hrs dark) and temperatures above 20°C. Upon transition to reproductive growth, the apical meristem and later also the lateral

Figure 1

meristems start to expand and eventually turn into groups of inflorescences (spikelets), each containing 3 to 10 floral meristems. The spikelets are attached alternately and directly to the central axis (rachis) (Figure 1B). Each floret consists of four whorls of organs. The outermost whorl (1) consists of the palea and the lemma both surrounding the lodicules (whorl 2), the three stamens (whorl 3) and the gynoecium (whorl 4) which is interpreted as syncarpous, consisting of two or three carpels forming the ovary (Figure 1C). The latter arises at the apex as a single ring-like structure surrounding the emerging single ovule (Barnard, 1957)

The flowers of the ryegrass inflorescence are arranged in a cymose, always terminating apical growth with the production of a terminal flower. In this way ryegrass and other grasses represents a determinate plant architecture also seen and described at the molecular level in dicot plants such as tobacco (Amaya *et. al.*, 1999) and tomato (Pnueli *et al.*, 1998). In contrast, plants such as *Arabidopsis* and *Antirrhinum* have an indeterminate (racemose) inflorescence. The *TERMINAL FLOWER 1 (TFL1)* gene of *Arabidopsis* and its homolog *CENTRORADIALIS (CEN)* in *Antirrhinum* have been identified as a group of genes that specify an indeterminate identity of inflorescence meristems. Mutations in *TFL1/CEN* result in the conversion of the inflorescence into a terminal flower (Shannon and Meeks-Wagner, 1991; Alvarez *et al.*, 1992; Bradley *et al.*, 1996, 1997; Ohshima *et al.*, 1997). In addition to its effect on meristem fate, *TFL1* also extends the V phase of *Arabidopsis* (Shanon and Meeks-Wagner, 1991), but *CEN* does not seem to have a flowering time role in *Antirrhinum* (Bradley *et al.*, 1996). CEN and TFL1 proteins have sequence similarity with mammalian phosphatidylethanolamine-binding proteins (PEBPs). These mammalian proteins were originally named for their ability to bind phospholipids *in vitro* (Grandy *et al.*, 1990), but have recently been demonstrated to be regulators of the central Raf1/MEK/ERK signaling pathway (Yeung *et al.*, 1999). The *FLOWERING LOCUS T (FT)* gene, also belongs to the family of plant PEBP genes, but has been shown to play an opposite role of *TFL1* in mediating flower inducing signals in *Arabidopsis* (Kardailsky *et al*, 1999; Kobayashi *et al*, 1999).

Antagonistically to the *TFL1* gene, another group of genes specify a determinate floral meristem identity. Well characterized genes such as *LEAFY (LFY), APETALA1 (AP1),* and *CAULIFLOWER (CAL)* from *Arabidopsis* belong to this group (Irish and Sussex, 1990; Schultz and Haughn, 1991; 1993; Mandel *et al.*, 1992; Weigel *et al.*, 1992; Bowmann *et al.*, 1993; Gustafson-Brown *et al.*, 1994). Mutations in either *LEAFY* or *AP1* result in replacement of floral meristems by shoot meristems, and in accordance with their role, overexpression of *LFY* or *AP1* in *Arabidopsis* converts shoots into flowers (Mandel and Yanofsky *et al.*, 1995; Weigel and Nilsson, 1995). Studies of expression patterns and combined analysis of mutants and overexpressing lines have

clarified some of the interactions among meristem identity genes (Levy and Dean, 1998, Nilsson *et al.*, 1998; Liljegren *et al.*, 1999; Ratcliffe *et al.*, 1999; Ferrándiz *et al.*, 2000; Samach *et al.*, 2000).

Antagonism between *TFL1* and *LFY, AP1* and *CAL1* is reflected in their complementary expression patterns and phenotypic effects. In wild-type *Arabidopsis* plants, *TFL1* is expressed in the center of the subapical region, whereas *LFY, AP1* and *CAL* expression are confined to the developing flowers (Mandel *et al.*, 1992; Bradley *et al.*, 1997; Ratcliffe *et al.*, 1999). In the *tfl1* mutant, the premature conversion of the apex into a floral meristem has been shown to be correlated with ectopic expression of *LFY* and *AP1/CAL* in the apex (Weigel *et al.*, 1992; Bowman *et al.*, 1993; Gustafson-Brown *et al.*, 1994; Bradley *et al.*, 1997). In accordance with this data, delayed upregulation of *LFY* and *AP1* is observed in 35S*TFL1* plants resulting in an extended vegetative phase (Ratcliffe *et al.*, 1998). There is increasing evidence to suggest that the ratio of *LFY/TFL1* activity in the SAM controls the developmental fate of the meristem (Ratcliffe *et al.*, 1999; Ferrándiz *et al.*, 2000). Thus, the lower the ratio, the longer the V phase will continue and the longer the SAM retains an indeterminate phenotype producing shoot meristems in place of flowers.

In order to investigate the mechanism underlying flowering control and plant architecture in a widely distributed, agronomically important monocot crop plant, we have isolated a homologue of the *Arabidopsis TFL1* gene from perennial ryegrass, *LpTFL1*, and characterized it for its potential role in determining plant architecture and the vegetative-reproductive phase transition in grasses. Our results suggest that *LpTFL1* is a repressor of flowering in ryegrass with a unique expression pattern not reported before. Overexpression of *LpTFL1* in *Arabidopsis* results in a dramatic extension of the V-I phase and a lateral branching that is consequently more extreme compared to overexpression of *TFL1* in *Arabidopsis*.

RESULTS

Isolation of a *TFL1*-like gene from ryegrass.

A 180 bp fragment with sequence similarity to *Arabidopsis TFL1* was amplified from ryegrass inflorescence mRNA by RT-PCR using primers designed on the basis of an alignment of *Arabidopsis TFL1*, *Antirrhinum CEN*, tomato *SP*, and a related rice EST (Figure 2A). This fragment was used to screen a ryegrass

Figure 2

flower cDNA library at moderate stringency for *TFL1*-like genes (*LpTFL1*-genes). One full-length cDNA was identified. The coding region of this cDNA shows 87% and 85% DNA sequence identity to two rice genes, *FDR2* and *FDR1*, and 67% and 64% identity with *TFL1* and *CEN*, respectively. The region in the rice EST used to design the *LpTFL1* specific primers is 86% identical to *LpTFL1*. On the protein level, LpTFL1 shows 91% and 86% identity to the corresponding proteins, FDR2 and FDR1, respectively, and 71% and 68% identity to TFL1 and CEN (Figure 2B). The *LpTFL1* cDNA coding region shows 60 % identity with the *FT* sequence, and the protein identity is 56%. Comparison of LpTFL1 sequence with other plant PEBP sequences found in the database revealed that LpTFL1 groups together with the two rice proteins and also CET1 from tobacco (Figure 2C). Banfield and Brady (2000) have recently determined the three-dimensional structure of the *CEN* protein and identified the amino acids essential for a functional ligand binding site. Other amino acids important for a functional protein have been identified by mutation (Bradley *et al*, 1997; Ohshima *et al.*, 1997; Pnueli *et al.*, 1998) (Figure 2B). Of these 11 amino acids, LpTFL1 differs from CEN at only one position (110) having a serine (Ser) instead of a methioine (Met).

A DNA blot analysis at moderate stringency using a full-length *LpTFL1* cDNA fragment as probe was performed to assess the number of *TFL1*-like genes in *L. perenne*. The results indicate that two *TFL1*-like genes are present in ryegrass (data not shown). In order to gain more information on the *LpTFL1* gene, we screened a ryegrass genomic library with the full-length *LpTFL1* cDNA clone. Three independent genomic clones were retrieved and sequenced. All had an identical DNA sequence predicting the same open reading frames (ORFs), which exactly matched the *LpTFL1* cDNA. The genomic organization of *LpTFL1* (Figure 2A) is similar to that of the *TFL1* and *CEN* with three introns in the same positions (Figure 2B) although the introns are of different sizes in ryegrass (100, 208, and 82 bp) compared to *Arabidopsis* (209, 205, and 86bp). In the ~3.6 kb region upstream of the transcription start no likely gene encoding ORFs were found, and therefore we assume this to be the *LpTFL1* promoter. The existance of regulatory elements further upstream the ~3.6 kb fragment cannot be excluded, however, in *Arabidopsis* only a ~2.7 kb non-coding DNA fragment is found upstream the transcription start of *TFL1* .

The *LpTFL1* gene in ryegrass shows an expression pattern different from *TFL1* in *Arabidopsis*.

To determine the expression pattern of *LpTFL1* message in ryegrass, we examined the mRNA levels in different tissues by a RNase protection assay. Ryegrass apices however, are extremely small (< 0.5 mm) until the

secondary induction stage, where the inflorescence rapidly expands, so in addition we also performed RT-PCR on apex samples that were excised from plants at different stages during flowering induction. Each reaction was repeated three times independently.

LpTFL1 message was detectable in most types of tissue, except for seeds (Figure 3A). As early as seedling stage (1-2 visible leaves) *LpTFL1* message was detectable by the RNase protection assay. Expression of *LpTFL1* was also detected in leaves from secondary induced plants, and it reached approximately the same level as *LpTFL1* message in the mature flowers. Leaf expression of *TFL1* in *Arabidopsis* has not been reported (Bradley *et al.*, 1997), however, detection of *CEN* message in *Antirrhinum,* and *CET1* message in tobacco in other tissues than the meristematic regions by RT-PCR have been reported (Bradley *et al.*, 1996; Amaya *et al.*, 1999). In the apex region, we found that *LpTFL1* message follows a bi-phasic expression pattern during the flowering induction. After 6 weeks of vernalization significantly less *LpTFL1* message was detected in the apex of vernalized plants compared to unvernalizeded plants (Figure 3B). In contrast, we could detect a continuous upregulation of *LpTFL1* message in the apex after the ryegrass plant had been transferred to the warmer conditions and LD photoperiods, which induce reproductive development. However, the level of *LpTFL1* message was lower in fully matured inflorescences (42 days sec. induction) compared to the stem and also the root (Figure 3A). A high level of *LpTFL1* transcription was detected in roots from plants growing in LD for 4 weeks.

Figure 3

LpTFL1 delays or prevents flowering in Arabidopsis

In order to address if *LpTFL1*, one of the two *TFL1*-like genes in ryegrass, has a function similar to the *Arabidopsis TFL1* we used the maize ubiquitin promoter (Christensen and Quail, 1996) to drive overexpression of the *LpTFL1* coding region in *Arabidopsis*. Following transformation with UBI::*LpTFL1*, 33 BASTA-resistant Arabidopsis plants were obtained. All the transformants showed remarkable vegetative characteristics and were much delayed in flowering compared to the wild-type (Figure 4 and 5). Whereas wild-type plants bolted 10 days after they were moved from SD to LD photoperiod, even the earliest flowering UBI::*LpTFL1* plants required another month in LD before they bolted. After 3 months, more than half of the plants had not produced a single flower (Figure 4C). Overexpression of *LpTFL1* affected both the vegetative and the early inflorescence stage of *Arabidopsis* as observed by the increased number of nodes produced both before and after bolting. During the

Figure 4
Figure 5

vegetative phase wild-type *Arabidopsis* plants produced 16 ± 1.9 rosette leaves, whereas the UBI::*LpTFL1* plants grown under the same conditions produced 33.9 ± 8.9 rosette leaves (not shown). After the plants had bolted, the UBI::*LpTFL1* plants produced 26 ± 14.3 cauline leaves on the main stem before flowering, in contrast to the wild-type which produced only 4.8 ± 0.4 cauline leaves (Figure 4C). Thus, both in terms of time and the number of nodes produced before flowering the majority of the UBI::*LpTFL1* plants appeared to be arrested in the early inflorescence phase. Similar observations were made in *Arabidopsis* plants in which overexpression of *TFL1* was driven by the 35S CaMV promoter (Ratcliffe *et al.*, 1998). However, the 35S::*TFL1* plants produced only two thirds of the number of rosette leaves and half of the number of cauline leaves compared to the UBI::*LpTFL1* plants, when grown under continuous light (Ratcliffe *et al.*, 1998). Five UBI::*LpTFL1* plants (lines 2, 7, 9, 11, and 13) remained in the early inflorescence stage throughout their life cycle and failed to produce flowers before they senescenced and died (after 7 month). Non-flowering individuals has also been observed in 35S::*TFL1 Arabidopsis*, however only when these plants were grown under SD condition (Ratcliffe *et al.*, 1998) and not under LD conditions, as reported here.

In addition to the main SAM, *Arabidopsis* plants transformed with UBI::*LpTFL1* also exhibit abnormal axillary meristem development. The development of coflorescences with developing flowers in the axils of the cauline leaves normally observed in wild-type *Arabidopsis* was rarely seen in the UBI::*LpTFL1* plants. However, in the place of floral organ formation, a 'leafy' branch was produced resulting in a highly branched, bushy, and dramatic phenotype (Figure 5A). Third-order branching was a common trait among the UBI::*LpTFL1* plants, and in a single plant also fourth-order branching was observed (Figure 5A right-hand plant). A reiterative series of leaves was continuously produced from the SAM of the UBI::*LpTFL1* plants, most of them with a high density of trichomes (Figure 5B). The trichome distribution on the surface of the cauline leaves was in general much more dense than in the wild-type (Figure 5C-D). Increased trichome production in relation to *TFL1* overexpression in *Arabidopsis* has not previously been reported. Compared with the wild-type, most of the UBI::*LpTFL1* plants produced remarkably more and longer internodes both on the main stem but also on the coflorescences. In contrast to the wild-type plants, the uppermost coflorescences without the subtending cauline leaf of the UBI::*LpTFL1* plants did not consist of normal solitary flowers but instead a leaf-like shoot (Figure 5E).

Based on the time to flowering, the transformants could be grouped into four classes (A-D) displaying a phenotype from late flowering (Figure 4C, group A) to never flowering (Figure 4C, group D). RNA gel blot analysis revealed that most of the UBI::*LpTFL1* plants showed strong expression of *LpTFL1* (Figure 4A, lines 1-

31). Overall, the severity of the UBI::*LpTFL1* plant phenotypes was positively correlated with the level of *LpTFL1* expression in the corresponding plants, although the two lines (1 and 23) with the highest *LpTFL1* expression level did flower after 174 and 182 days, respectively. The expression level of *LpTFL1* in turn, was positively correlated with the total number of gene copies inserted in the genome as determined by DNA gel blot analysis (data not shown). In plants with a single copy insertion (Figure 4A, lines 5, 16, 29-30), the *LpTFL1* RNA levels were reduced compared to other lines and consequently the phenotype was less severe but the time to flowering was still significantly longer than in the wild-type (Figure 4C group A). Three BASTA resistant plants in which *LpTFL1* expression was not detected by gel blot analysis, looked similar to wild-type plants with respect to their morphology, but flowered 10 days later than the wild-type (not shown).

Detection of the floral meristem identity genes *AP1* was performed by RT-PCR on mRNA isolated from *Arabidopsis* wild-type, the *tfl1-14* mutant, and the hemizygote second generation UBI::*LpTFL1* line 17. The plants were harvested when the mutant flowered and the first floral organs were visible in the wild-type. *AP1* transcript was detectable both in the *tfl1-14* mutant and the wild-type, but was not detectable in the UB:*LpTFL1* line 17 (Figure 4C) suggesting that *LpTFL1*, like *TFL1*, is capable of suppressing or delaying the transcription of *AP1* in *Arabidopsis* .

LpTFL1 overexpression in a _tfl1-14_ mutant background

In order to further address the functional similarity between LpTFL1 and *Arabidopsis* TFL1 we asked if LpTFL1 is able to complement the *Arabidopsis tfl1-14* strong mutant allele. In this mutant a C to T mutation leads to a threonine → isoleucine substitution at position 69 (Figure 2B). The *tfl1-14* mutant has a short vegetative phase and exhibits reduced plant height with few nodes, increased number of inflorescence arising from the rosette axillary meristems, and a determinate growth pattern (Bradley *et al.*, 1997; Ohshima *et al.*, 1997). The construct used for transformation of the *Arabidopsis* wild-type was also used for transformation of the *tfl1-14* mutant. More than 100 independent UBI::*LpTFL1-tfl1-14* primary transformants were obtained from each mutant line after selection for the binary plasmid. All the plants displayed a variety of phenotypes from wild-type to the same extended vegetative phenotype seen in the UBI::*LpTFL1* wild-type background. On average (taken only from the first six plants flowering) the UBI::*LpTFL1-tfl1-14* plants produced 15.2 ± 3.5 cauline leaves on the main stem and flowered 33 days later than the *tfl1-14* mutant and 23 days later than the wild-type (Figure 4C). All the UBI::*LpTFL1-tfl1-14* plants grew indefinitely and the production of terminal

flowers and rosette inflorescence, which is always seen in the *tfl1-14* mutants, was never observed in the transformants. Thus, the *LpTFL1* rescued the *Arabidopsis tfl1-14* mutant in terms of morphology, and the extended vegetative appearance we observe is presumably due to the force of the relatively strong maize ubiquitin promoter.

***LpTFL1* is a potential regulator of axillary meristem development.**

We examined the properties of the ~3.6 kb putative *LpTFL1* promoter by fusing it to the *Uid*A gene and transformed it into *Arabidopsis*. More than 100 BASTA resistant lines were obtained. Ten seedlings of primary transformants with 6-7 rosette leaves were tested for GUS expression. Three out of the ten transformants showed GUS expression, and in all three plants the expression was confined to a very narrow area in the axillary meristems of the rosette leaves (Figure 6A). Another test for GUS activity was performed after the plants had bolted and had produced 4-5 cauline leaves with flowers in the axils. Fifteen primary transformants were tested, and GUS activity was detected in three of these plants. At this stage GUS activity was still detected in the axillary meristems of the rosette leaves but it was also detected in the axillary meristems of the cauline leaves, although weaker (Figure 6B). In this area, however, GUS-activity remained even after the formation of a new flower (Figure 6B). No GUS expression was detected in the apical meristem or in *Arabidopsis* leaves, although *LpTFL1* is expressed in ryegrass leaves. Thus, we find that the activity of the ~3.6kb *LpTFL1* promoter used in this experiment is regulated differently in *Arabidopsis* than in ryegrass, and the pattern of GUS-activity is very similar to the expression patterns of *CET2* and *CET4* in tobacco (Amaya *et al.*, 1999). These genes are reported to be expressed only in the axillary meristems and not in the SAM.

DISCUSSION

Perennial ryegrass is a forage grass with a high agronomic value, since it is a low-cost crop, it is perennial and it is widely used for feeding cattle. One of the major goals in crop improvement is the control of reproductive growth and flower development. Molecular information on these events is very limited in this species. We have isolated a *TFL1*-like gene from perennial ryegrass, which is likely to be a repressor of flowering and involved in control of axillary meristem identity.

Lolium LpTFL1 is a new member of the plant PEBP family

The *Lolium perenne TFL1*-like gene, *LpTFL1*, encodes a protein with high homology to a group of plant proteins which share structural similarities to mammalian phosphatidylethanolamine-binding proteins (PEBPs). Based on these similarities, the plant PEBPs are predicted to play a role in the regulation of signaling cascades as has been shown for the mammalian PEBPs (Banfield and Brady, 2000; Young *et al.*, 1999). The two proteins most similar to LpTFL1 are the rice FDR2 and FDR1 with 91% and 86% identity, respectively. In a multiple comparison including TFL1-like proteins from different species as well as FT from *Arabidopsis*, LpTFL1 is grouped together with the two rice proteins and a tobacco CEN-like protein, CET1. No data on *FDR2/ FDR1* expression patterns and functions in rice has been reported, and for *CET1*, expression has been reported to be detectable both in vegetative and inflorecence shoots, but only by RT-PCR (Amaya *et al.*, 1999). Compared with the *Arabidopsis* PEBP sequences, LpTFL1 shows 71% identity to TFL1 and 56% identity to FT. FT, which is 56% identical to the TFL1 protein, also belongs to the family of plant PEBPs, however, in contrast to TFL1, FT has been shown to mediate flowering inducing signals in *Arabidopsis* (Kardailsky *et al.*, 1999; Kobayashi *et al.*, 1999). In this process FT acts in parallel with and under the influence of the *CONSTANS (CO)* gene, which is a mediator of the LD-induction pathway (Samach *et al.*, 2000). LpTFL1 shows 50% identity to a partial *FT*-like region on a rice clone (nbxb0035E07r), but although the DNA blot analysis indicates the existence of another *LpTFL1*-like gene, no ryegrass *FT*-like cDNA with a higher homology to this partial rice *FT*-like sequence has been identified yet. Overexpression of *LpTFL1* in *Arabidopsis* results in significantly delayed flowering in combination with a dramatic large and bushy phenotype, suggesting that LpTFL1 is more TFL1-like than FT-like.

In spite of the high degree of homology between the plant PEBPs, constitutive expression of these proteins in different plants leads to different phenotypes. The dramatic impact of *LpTFL1* overexpression on floral transition and plant architecture in *Arabidopsis* is more extreme than that previously reported by overexpressing *TFL1* in *Arabidopsis* (Ratcliffe *et al.*, 1998). One possible explanation for the more severe phenotype observed in our study may be that the activity of the maize ubiquitin promoter is stronger than the 35S CaMV promoter in *Arabidopsis*. If this is the case, the monocot ubiquitin promoter shows a remarkably strong activity not previously reported in a dicot plant. Alternatively, our observation may be due to differences in the protein sequence and conformation of LpTFL1 compared to TFL1. Overexpression of *CEN* in tobacco has also been reported to significantly delay the floral transition as well as changing the plant architecture (Amaya *et al.*, 1999). In contrast, there was no effect of overexpressing *TFL1* in tobacco (Amaya *et al.*, 1999). These results

together with our results indicate, that differences in the protein sequences among the plant PEBPs are likely to account for some of the differences observed in the overexpressing plants.

Eleven amino acid residues in the plant PEBP sequences have so far been identified as essential for a functional protein (Figure 2B) either by crystallography (Banfield and Brady, 2000) or by mutations (Bradley *et al.*, 1997; Ohshima *et al.*, 1997; Pnueli *et al.*, 1998). At theses residues, LpTFL1 differs from the consensus at one position (110) which is also the position with the highest degree of amino acid variation between species. Interestingly, the variation in amino acid residues at position 110 exactly matches the grouping of plant PEBP by the clustalW alignment (except for FT). One group comprising TFL1, BNTFL1-1, and BNTFL1-3, has a leucine at this position, another group, comprising CEN, CET2, CET4, and SP, has a methionine, and a third group, which includes LpTFL1, FDR2, FDR1, and CET1, has a serine at this position. The immediate assumption that the amino acid differences at this position can be linked to the variance in phenotype servereness of plants overexpressing different *TFL1*-like genes, would suggest that overexpression of BNTFL1-1/BNTFL1-3, like TFL1, also have no effect in species like tobacco, and that overexpression of CET1, FDR2/FDR1, like LpTFL1 might have a significant effect on plant architecture and flowering time in species like *Arabidopsis*. Future results on overexpression of *TFL1*-like genes in different species would contribute to resolve the correlation between protein sequence and the effect on morphology. In any case our results show that the effects of different PEBPs can not solely be explained by genetical diversity, since ryegrass is more distantly related to any of the dicot species, and yet LpTFL1 has a strong and unequivocal effect on the *Arabidopsis*.

Control of floral transition.

The dramatic phenotype of *Arabidopsis* plants overexpressing *LpTFL1* suggests that, in ryegrass, *LpTFL1* may play a role both in controlling meristem identity and in the transition from vegetative to reproductive growth. In ryegrass, *LpTFL1* message is detected at all stages from germination to maturity. It is found at the apex, in the inflorescence, and also in leaves, stems, roots and mature flowers. However, expression of *LpTFL1* in the ryegrass apex is not constitutive. Levels of *LpTFL1* message change during flower induction, with a significant downregulation after 6 weeks of vernalization, followed by a continuous upregulation during secondary induction until the structures of the spikelets are visible. Data on cold-induced downregulation of *TFL1*-like genes in other species has not been reported, presumably because vernalization is normally not required for flowering induction in the tested species. Analysis of the expression pattern of *FLOWERING*

LOCUS C (FLC) in *Arabidopsis*, a gene encoding a MADS-box repressor of flowering that is downregulated by vernalization (Sheldon *et al.*, 1999), suggests that the physiological role of vernalization is to alleviate a block in flowering. Since *LpTFL1* may function as a repressor of flowering in perennial ryegrass, a species which requires at least 12 weeks of cold treatment in order to flower, downregulation of *LpTFL1* during vernalization may be necessary in order for ryegrass to proceed to the reproductive phase. Intriguingly, the late-flowering phenotype of 35S::*TFL1 Arabidopsis* can be suppressed by a vernalization treatment, suggesting that *TFL1* may repress the upregulation of floral meristem identity genes by increasing FLC activity or expression (Simpson *et al.*, 1999; Ratcliffe *et al.*, 1998). In unvernalized perennial ryegrass an analogous system may be operating in which the level of a FLC-like protein might be high due in part to induction by *LpTFL1*. Downregulation of *LpTFL1* upon vernalization, and perhaps an FLC-like protein as well, would then be necessary in order for the meristem to be competent to flower.

Some of the *Arabidopsis* plants overexpressing *LpTFL1* never flowered before senescence. Similar vegetative non-flowering *Arabidopsis* plants were obtained by combining mutations in *AP1, CAULIFLOWER (CAL)* and *FRUITFULL (FUL)*, all three MADS-box genes (Ferrándiz *et al.*, 2000). The vegetative growth of the triple mutant was correlated with a low *LFY:TFL1* transcript ratio and with *TFL1* becoming ectopically expressed in the laterally arising meristems in an overlapping pattern with *LFY* expression. The SAM of the *ap1, cal, ful* triple mutant is arrested in the V→I_1 phase producing only cauline leaves with axillary meristems that in turn repeat this pattern forming 'leafy' cauliflower along the main inflorescence (Ferrándiz *et al.*, 2000). Similar cauliflower-like structures were not observed in our UBI::*LpTFL1* plants because the repeated formation of meristems was slower. However, an additional morphological characteristic of the UBI::*LpTFL1* plants was the high density of trichomes which covered both the leaves and the SAM (Figure 5). In *Arabidopsis*, disappearance of the trichomes from the adaxial surface of cauline leaves has been shown to be tightly linked to floral induction (Telfer *et al.*, 1997) In support for this observation, it was shown that a mutation in *TFL1* leads to accelerated loss of adaxial trichomes in *Arabidopsis* (Telfer *et al.*, 1997) In agreement with this we find that the expression of a *TFL1*-like gene in *Arabidopsis* prevents the loss of adaxial trichomes. By this criterion the UBI::*LpTFL1* plants were less competent to flower compared to the triple mutant. However, the fact that most of the UBI::*LpTFL1* plants do flower, and produce normal flowers after an extended vegetative phase, suggests that a delayed but otherwise normal expression of *LFY, AP1, CAL* and the organ identity genes has occurred. Therefore, either the level of LpTFL1 activity can be decreased over time, or additional factors override LpTFL1 function and ensure the proper transcription of meristem and organ identity genes. One possible factor is FT,

which is able to upregulate floral meristem identiy gene like AP1 and *LFY* (Kobayashi *et al.*, 1999; Samach *et al.*, 2000).

A potential molecular mechanism for determinate plant architecture in perennial ryegrass.

Perennial ryegrass and *Arabidopsis* represent two different forms of plant architecture; determinate and indeterminate, respectively. A molecular basis for indeterminate growth has been proposed for *Arabidopsis* (Bradley *et al.*, 1996; 1997; Ratcliffe *et al.*, 1998; 1999; Liljegreen *et al.*, 1999) in which indeterminate plant architecture is correlated with expression of *TFL1* in the center of the SAM. In this central region as well as in the uppermost layers of the SAM, TFL1 activity is capable of excluding the expression of both *AP1* and *LFY*, and therefore the the formation of a terminal flower. Several parallels can be drawn between the A*rabidopsis* data and our results. Like *TFL1* in *Arabidopsis*, *LpTFL1* message is present in the ryegrass apex at the vegetative stage. Furthermore, a significant upregulation of *LpTFL1* occurs in the apex upon LD induction, which is similar to the upregulation of *TFL1* observed in *Arabidopsis* when the plants enter the I_1 phase (Ratcliffe *et al.*, 1999). Like *TFL1*, overexpression of *LpTFL1* results in a repression of *AP1* transcription (Figure 4C). Localization of *LpTFL1* expression in the ryegrass apex awaits characterization, but our initial data based on the analysis of the *LpTFL1* promoter driving GUS expression in *Arabidopsis* showed strong activity in the axillary meristems of the rosette leaves during the vegetative phase (Figure 7A). At later stages, when plants had bolted, GUS activity was still detectable in the rosette axillary meristems but also in the axillary meristems of the cauline leaves (Figure 7B), although weaker. No GUS activity was detected in the SAM where *TFL1* is normally expressed. The lack of expression in this region points to the posibility that either some regulatory elements are missing in the ~3.6 kb *LpTFL1* promoter fragment, or that the elements in the monocot promoter is not fully recognized in *Arabidopsis*. Indeed, Oshima *et al.* (1997) found that a T-DNA insertion into a site located 458 bp downstream of the putative *TFL1* polyadenylation signal lead to an *Arabidopsis tfl1* mutant phenotype, suggesting the existence of a regulatory element in this area. However the *LpTFL1* driven GUS expression pattern in *Arabidopsis* is similar to the expression pattern of *CET2/CET4* in tobacco. Like ryegrass, tobacco is a species with determinate plant architecture, and seven *CEN/TFL1*-like genes have been isolated from this plant (Amaya et al., 1999). Expression of the tobacco genes most similar to *CEN*, (*CET2* and *CET4*) has been shown to be restricted to the axillary meristems, and the determinate growth of the tobacco SAM has been correlated with the absence of *CET2/CET4* expression from the SAM (Amaya *et al.*, 1999). Consistent with this, Amaya *et al.*

(1999) found that *NFL*, the *LFY* homolog is strongly expressed in the inflorescence apical meristem. A similar *AP1- LFY* –like mediated exclusion of *LpTFL1* from the center of the ryegrass SAM still needs to be confirmed. Pollen of *Arabidopsis LpTFL1*::GUS plants is currently being crossed to the *Arabidopsis tfl1-14* mutant in order to adress this question. However, we do propose that in the meristematic ridges of the ryegrass apex (the axillary meristems), *LpTFL1* expression might serve to repress the transcription of floral identity genes and specify a shoot meristem identity.

Analysis of transgenic ryegrass overexpressing *LpTFL1* is in progress, and based on the data presented here we can speculate the following scenario for *LpTFL*-mediated control of floral transition and plant architecture: Shortly after germination, *LpTFL1* expression is established in the meristematic ridges of the apex to maintain the production of vegetative organs such as leaves and tillers. During vegetative growth, a high level of *LpTFL1* and other flowering repressors, perhaps similar to *FLC*, is maintained to avoid precocious flowering before the winter season. During the winter vernalization period, levels of *LpTFL1* and other flowering repressors decreases allowing the plants to become competent for flowering. As the temperature increases and the photoperiods lengthen in spring, *LpTFL1* expression is upregulated in the arising spikelets on the flanks of the apex in order to promote lateral branching of the main axis. In this way a maximum number of spikelets are produced. Subsequently, expression of *LpTFL1* becomes progressively more restricted to vegetative tissues such as stem and root, and the ryegrass plant finishes its life cycle by the production of the last uppermost seed in the top spike.

Dissection of the molecular mechanisms underlying the floral transition and flower formation in important monocot crop plants like perennial ryegrass is in its infancy. By analyzing one of the main components in this multi-factorial process, a *TFL1*-like gene in perennial ryegrass, we have just begun to adress the question of how the green common ryegrass growing on millions of acres, is transformed into flowering plants each year following winter season. Further studies will perhaps confirm and certainly modify the scenario for *LpTFL1* function that we propose here.

MATERIALS AND METHODS

Plant growth conditions

Ryegrass (*Lolium perenne* L.) plants (clone F6, DLF Trifolium) were grown in soil in a greenhouse with daylight at 21 and 18°C, day and night temperature, respectively. For the primary induction (vernalization) plants were grown in a growth chamber at or below 5°C for at least 12 weeks. During vernalization, the light period was decreased to 8 hrs per day. Following vernalization, plants were grown under 16 hrs light at 22 and 18°C, day and night temperature, respectively for secondary induction. For RNA analysis, plants were harvested before vernalization, after 6 weeks of vernalization, and after 14 and 28 days of secondary induction and meristems were excised. Samples from other tissues like leaves, stems, seeds and roots were also harvested for expression analysis.

Arabidopsis seeds were stratified for 2-3 days at 4°C and then grown in soil in growth chambers at 22 and 18°C, day and night temperature, respectively. During the first two weeks, plants were grown at short day conditions (8 hours. light per day) and then moved to long day conditions (16 hours light per day). In the *Arabidopsis* time-course experiment, rosette leaves were counted when plants started to bolt, and the number of leaf nodes were counted from the most basal cauline leaf to the uppermost leaf proximal to the inflorescence. The number of days from germination to the production of the first flower-like structure, was also scored.

Screening of cDNA and genomic library.

To isolate *TFL1*-like genes from ryegrass, a set of primers partially conserved between *TFL1* of *Arabidopsis*, *CEN* of *Antirrhinum* and a rice EST (RICR2918A; accession: 428842) were designed. Primer RY2N (5'-GGTTATGACAGACCCAGATGTG-3') was used in combination with primer RY4V (5'-CGAACCTGTGGATACCAATG-3') to amplify a 180 bp fragment by RT-PCR. Preparation of RNA for the RT-PCR used the FastRNA®, GREEN Kit RNA isolation system (Bio101). The 180 bp fragment corresponding to a putative ryegrass *TFL1*-like fragment was used to screen a cDNA library (Stratagene) made of ryegrass inflorescences for full-length *TFL1*-like cDNAs. Approximately 800,000 recombinants were screened at moderate stringency of 60°C, with washes at 60°C in 2 X SSC (0.3 M NaCl and 0.1 M sodium citrate, pH 7.4) and 0.1 % (w/v) SDS. Three positive clones were isolated, and plasmids were isolated from single plaques by *in vivo* excision. All cDNA clones were sequenced and contained identical sequences with similarity to both *TFL1* and *CEN* and were named *Lolium perenne TFL1*-like, *LpTFL1* (genebank accession number: AF316419).

A λEMBL3 SP6/T7 genomic library (Clontech) made from a partial *Sau3A* digest of ryegrass DNA was screened for *TFL1*-like genes. Approximately 1,000,000 recombinants were screened at moderate stringency (as described above) with the full-length *LpTFL1* cDNA clone. Nine positive clones were isolated and digestion of the λ DNA clones with *BamHI, SalI, XbaI,* and *SacI* revealed that three unique clones. These clones were partially sequenced and all three had identical sequence from 4.0 kb upstream and 2.0 kb. downstream the *LpTFL1* sequence. The sequence of the exons of the genomic clones as well as the 5' and 3' untranslated region were identical to *LpTFL1*. DNA sequencing was performed using the ABI Prism system (Perkin-Elmer), and sequence analysis and alignments were produced using Gene Codes Sequencer software version 4.02.

RNA/DNA analysis

For detection of *LpTFL1* RNA level in different organs of ryegrass, 15 μg of RNA was hybridized to a 350 bp riboprobe corresponding to the 5' end of *LpTFL1* antisense. The riboprobe was synthesized by *in vitro* transcription from a T7 priming site fused to the 350 bp *LpTFL1* fragment by a T7 RNA polymerase in the presence of [α-^{32}P]CTP (800 Ci/mMol). Hybridization, RNase digestion and precipitation was performed as described in the system kit (RPA III™, Ambion) End products were electrophoresed on a 6% poly-acrylamide gel. The level of transcript was determined by autoradiography. A riboprobe corresponding to 180 bp of ryegrass *GAPDH* antisense fragment was used as control.

Changes in *LpTFL1* RNA levels in meristems during the flowering induction were detected by RT-PCR. Poly-A$^+$ mRNA was isolated from 5 μg of total RNA and all mRNA was used in the reverse transcription. Two internal primers INS5 (5'-CACATTGGTTATGACGGACC-3') and INS3 (CTCCCCCCCAAATGAAGC-3') were used in the subsequent PCR reaction to amplify a 200 bp *LpTFL1* fragment from the first strand cDNA templates. Products from 15, 20, and 25 PCR-cycles were electrophoresed, blotted and hybridized to a *LpTFL1* specific probe using standard blotting techniques. For a positive control, primers for ryegrass *ACTIN*: AC5 (5'-GAGAAGATGACCCARATC-3') and AC3 (5'-CACTTCATGATGGAGTTGT-3') were used. Detection of *LpTFL1* RNA levels in transformed *Arabidopsis* was performed by standard RNA gel blot analysis. Detection of *AP1* RNA levels in the 4 week old *tfl1-14* mutant, wild-type and the UBI::*LpTFL1* line 17 was performed by RT-PCR using two 3'-end specific *AP1* primers AP05 (5'-CCCCCTCTGCCACCG-3') and AP03 (5'-AGGTTGCAGTTGTAAACGGG-3').

Construction of UBI::*LpTFL1* and the transformation of *Arabidopsis* wild-type and *tfl1* mutants

The coding region of *LpTFL1* cDNA was amplified using primers B0 (5'-GGATCCCCATGTCTAGGTCTGTGGAG-3') and B550 (5'-GGGATCCCACAACTGGGATAGCCA-3') and recombinant *pfu* polymerase. The fragment was blunt ligated into vector pAHC27 (Christensen and Quail, 1996) containing the maize *Ubiquitin* promoter, an Exon:intron region and the NOS terminator. The entire cassette (UBI::EXintron::*LpTFL1*::NOS) was excised from the plasmid by digestion with *HindII* and *EcoRI* and ligated into the *EcoRI-HindII* site of the binary vector pCAMBIA3300 (Jefferson, Australia) which confers BASTA® resistance, to give pCAMLPTFL1. *Arabidopsis* plants (Columbia and *tfl1-14* mutants) were transformed with *Agrobacterium tumefaciens* strain GV3101 (Koncz and Schell, 1986) harboring the pCAMLPTFL1 (for *LpTFL1* overexpression) using the 'floral dip' method described by Clough and Bent (1998).

Construction of LpTFL1::*GUS Arabidopsis* plants

A 3.6 kb *SacI-RcaI* DNA fragment upstream the start codon of *LpTFL1* was ligated into the *SacI-NcoI* site of the binary vector pCAMBIA3301 (Jefferson, Australia) replacing the CaMV 35S promoter in front of the GUS first intron to give pCAMLpTFL1GUS. This vector also confers resistance to BASTA®. *Arabidopsis* plants (Columbia) were transformed with pCAMLPTFL1GUS and with the original pCAMBIA3301 using the method described above. The colorimetric X-gluc assay was used to determine the localization of GUS expression in *Arabidopsis* tissue (Jefferson, 1987). Leaves, stem, inflorescence and axillary meristems were vacuum infiltrated, and samples were incubated in the solution overnight at 37^{o}C in the dark. Tissues were bleached in 96% ethanol, and GUS expression was recorded with a Nikon stereo microscope.

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Figure 1: Comparative morphology of perennial ryegrass and *Arabidopsis*. A, The ryegrass vegetative SAM is very compact with semi-circular ridges that later will give rise to leaves and tillers. It is positioned on the basal plate and surrounded by developing leaves. Bar = 1.0 mm. B, The ryegrass inflorescence consists of spikelets alternately attached to the main axis (rachis). Each spikelet consists of 3-10 flowers. Bar = 1.0 mm. C, Schematic diagrams of ryegrass and *Arabidopsis*. During vegetative growth, the SAM of ryegrass and *Arabidopsis* produce very closely spaced leaves in a rosette. After the floral transition the SAM of both species elongate (bolt) and floral organs (circles) are produced along the main axis. In both plants secondary shoots arise from the axils of subtending leaves. In *Arabidopsis* wild-type, flowers mature in an acropetal order and the SAM grows indefinitely (arrowheads), whereas in the *tfl1* mutant both the SAM and the secondary shoots terminate in a flower. Like the *tfl1* mutant, the ryegrass SAM and secondary shoots also terminate in a flower. Maturation of flowers in the ryegrass inflorescence is basipetal, and all the secondary shoots formed below the apex also develop into arrays of flowers in a cymose pattern. The collar is a special meristematic region on the leaf blade in the junction between the leaf blade and the stem (closed circles). An enlargement of a floret is shown

(redrawn from K. Esau, *Anatomy of seed plants*, 2nd ed. Wiley & Sons, Inc. 1977). Each floret consists of four whorls of organs. The outermost whorl consists of the palea and the lemma both surrounding the lodicules (whorl 2), the three stamens (whorl 3) and the ovary (whorl 4) which is interpreted as syncarpous, consisting of two or three carpels forming the ovary (Figure 1C). The latter arises at the apex as a single ring-like structure surrounding the emerging single ovule (Barnard, 1957)

Figure 2: Genomic organizataion of *LpTFL1*, and similarity of the deduced protein with other plant PEBPs. A, The upper bar shows the genomic organization of the gene including the untranslated (shaded boxes) and the translated (open boxes) regions. A 180 bp *TFL1*-like DNA fragment was isolated from ryegrass by RT-PCR. B, Comparison of the deduced protein sequence for the *LpTFL1* gene (acc. no: AF316419) with those of *TFL1* (Bradley *et al.*, 1997; Ohshima *et al.*, 1997), *CEN* (Bradley *et al.*, 1996), *SP* (Pnueli *et al.*, 1998), *BNTFL1-1*, *BNTFL1-3* (Mimida *et al.*, 1999), *CET1*, *CET2*, *CET4* (Amaya *et al.*, 1999), *FDR1*, *FDR2* (accession: AAD42896 and AAD42895, respectively), and *FT* (Kobayashi *et al.*, 2000; Kardailsky *et al.*, 2000). CLUSTAL W program was used to make the alignment and the deduced distance tree. Identical residues are in black. Dashed lines indicate gaps introduced by the program to achieve maximum alignment. Identical intron positions among all species are marked with filled arrowheads. Open arrowheads indicate amino acids identified to be at the ligand binding sites by crystallography (Banfield and Brady, 2000), and asterisks indicate amino acids in which point mutations were described for *Arabidopsis* (Bradley *et al.*, 1997; Ohshima *et al.*, 1997) and tomato (Pnueli *et al*, 1995). C, Distance tree of different plant PEBPs. The lengths of the horizontal lines are proportional to the similarity between the predicted protein sequences.

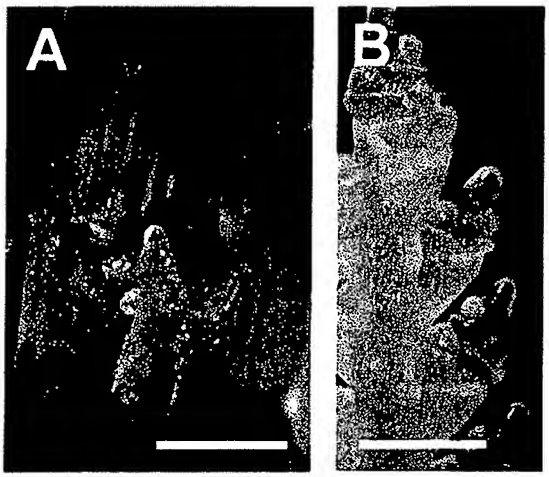
Figure 3: *LpTFL1* mRNA levels in various tissues detected by ribonuclease protection assay (A) and RT-PCR (B). 15 µg of RNA were used from each kind of tissue which were hybridized with a 350 bp *LpTFL1* and a 180 bp *GAPDH* antisense riboprobe before RNase digestion. For a positive control, *LpTFL1* antisense probe was incubated with yeast RNA. B, 5 µg of RNA from meristems and roots from different time-points during flowering induction were used for the RT-PCR.

Figure 4: UBI-*LpTFL1* dramatically alters the duration of the vegetative phase of *Arabidopsis*. A, RNA gel blot analysis of primary transformants (lines 1-30) and wild-type plants (WT). 15 μg of RNA from rosette leaves was blotted and probed with a *LpTFL1* cDNA probe. Transgenic line 5, 16, 29 and 30 have single copy insertion as detected by DNA blot analysis (not shown). Line 2, 7, 9, 11, and 13 were non-flowering. B, Expression of *AP1* and *ACTIN* in a *tfl1-14* mutant line, wild-type, and in a UBI::*LpTFL1* plant (line 17) as detected by RT-PCR on 5 μg of RNA from each plant. C, Number of cauline leaves produced on the main stem in *tfl1* mutant, the complemented mutant (*tfl1**), wild-type (WT) and UBI-*LpTFL1* primary transformants (group A-D). Each bar represents the mean value of the plants within the specific group. Numbers above the bar indicates the total number of days from germination till the onset of the first flower. The plants were grouped according to the time to flowering: (A ≥ 75 days; B ≥ 100 days; C ≥ 150 days; D non-flowering (NF)) The number of plants in each group is *tfl1*, 6; *tfl1**, 6; WT, 6; A, 6; B, 13; C, 5; D, 5.

Figure 5: The effect of UBI-*LpTFL1* on the morphology of *Arabidopsis*. A, The UBI::*LpTFL1* *Arabidopsis* primary transformants, line 1 and 2 (right-hand side), showing extensive vegetative growth and up to fourth order branching 4 months after germination compared with a 1 month old flowering wild-type plant (left-hand side). Line 2 (middle) was non-flowering after 7 months of growth. B, The SAM of most UBI::*LpTFL1* *Arabidopsis* lines is compact, filled with leaf primordia, and covered with trichomes. C and D, Trichome distribution on the adaxial surface of the uppermost cauline leaves on the main stem of UBI::*LpTFL1* (C) compared to wild-type cauline leaves at same age (D). E, In the UBI::*LpTFL1* plants leafy shoots filled with trichomes are produced in place of normal flowers on the upper coflorescences.

Figure 6: LpTFL1 promoter activity in *Arabidopsis*. A, Thirteen days post-germination, GUS activity is detectable in a confined area around the axils of the rosette leaves in LpTFL::*GUS* plants. B, At later stages (7 days after bolting), GUS activity is also detected in the axils of cauline leaves, although weaker

Figure 1: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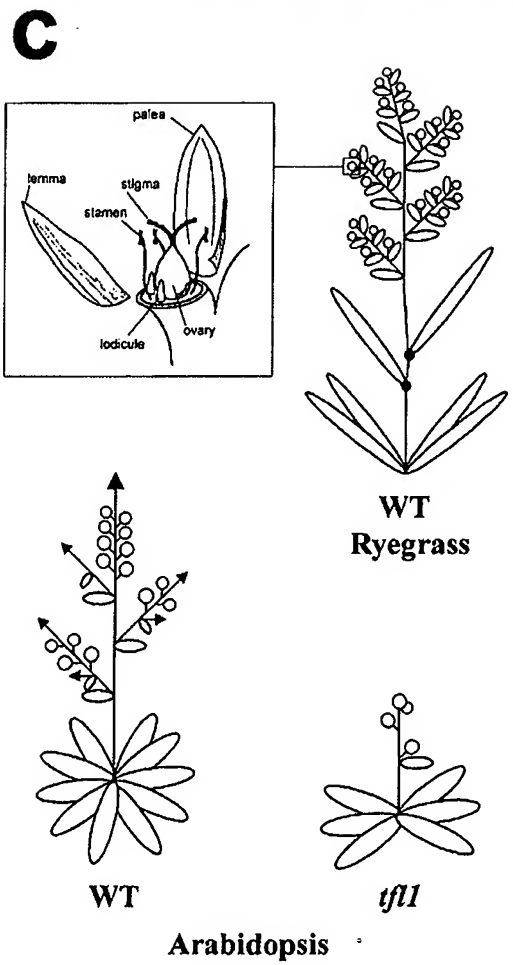
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Figure 2.

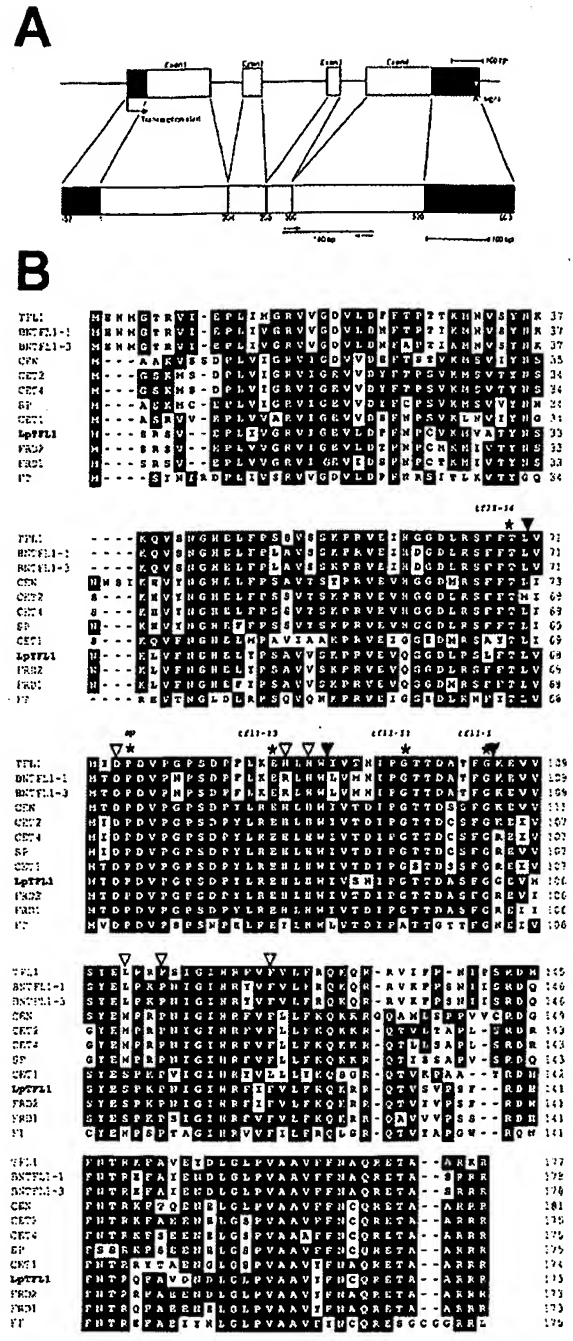
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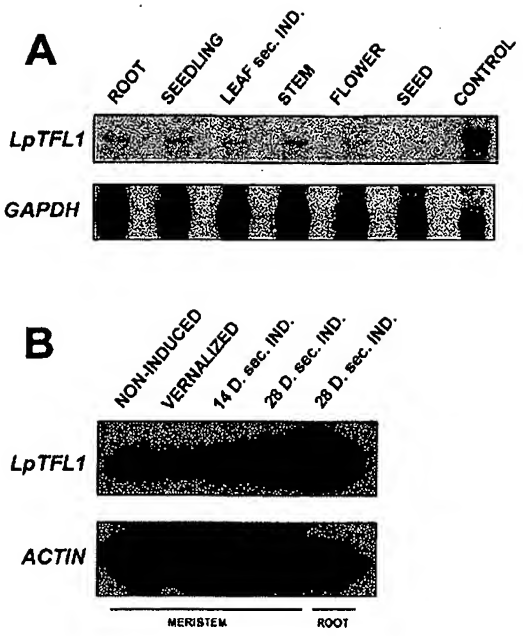
Figure 3.



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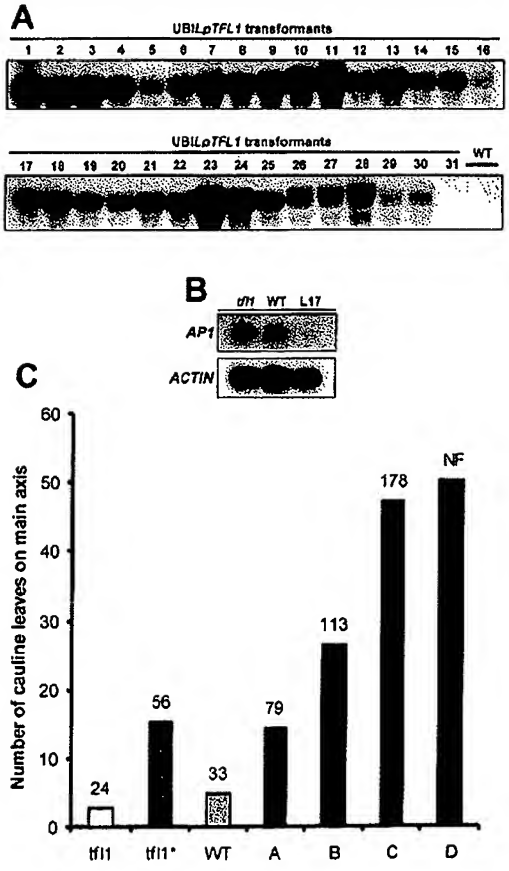

Figure 5.

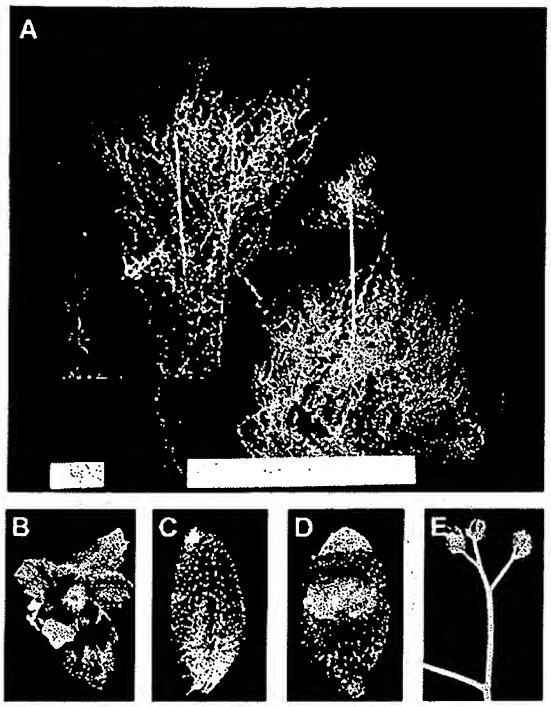

Figure 6.